

2016

Application of Advanced Analytical Technologies to Drug Development Studies and Cancer Detection

Ramakrishna Reddy Voggu

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**APPLICATION OF ADVANCED ANALYTICAL TECHNOLOGIES TO DRUG
DEVELOPMENT STUDIES AND CANCER DETECTION**

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May 2010

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DOCTOR OF PHILOSOPHY IN CLINICAL-BIOANALYTICAL CHEMISTRY

At

CLEVELAND STATE UNIVERSITY

August 2016

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ACKNOWLEDGEMENT

I would like to express my appreciation to all my mentors, friends, and family members for continuous guidance and support towards the completion of this work in this long journey.

My deepest gratitude goes to my research advisor Dr. Baochuan Guo for giving me this wonderful opportunity, who always believed in me and encourages me when I am facing challenges. His invaluable advice and insight have had enormous influence on my work. Additionally, he provided me with many opportunities to facilitate my ability in independent research and my development as a professional. I wish to thank him for his kindness and the rewarding experience I have had in the past four years.

I would like to thank my committee members. I thank Dr. Xiang Zhou for training me on instrumentation, offering me a great opportunity to work as an instrumentation assistant. I also would like to thank Dr. Bin Su for allowing me to collaborate on his key projects and also in spending time with me to further improve my writing skills. Also, I would like to thank Dr. Amin Zhou and Dr. Kothapalli Chandra, for giving precious suggestions on my research. I admire their insight and expertise from different areas, which inspired me in developing many ideas through my research.

Special thanks to my lab members. I enjoy the friendly working environment and I deeply appreciate their technical help and valuable discussion. Also, I would like to thank Prasad Gobburi, Shashank Gorityala and Anvesh Dasari for their invaluable support both personally and professionally.

Finally, I would like to express my deepest regards to my Mom, Dad, Sister, Brother-in-law, Grand Father and each and every member of my family who has supported and stood by me at each and every step of my career.

APPLICATION OF ADVANCED ANALYTICAL TECHNOLOGIES TO DRUG DEVELOPMENT STUDIES AND CANCER DETECTION

RAMAKRISHNA REDDY VOGGU

ABSTRACT

In the past decade, bioanalytical method development has become an integral part of the clinical diagnosis, biomarker discovery, and drug discovery and development. The new and emerged bioanalytical techniques allow the quantitative and qualitative analysis of bio molecules with remarkably high sensitivity and specificity. Specifically, these bioanalytical methods based on LC-MS and methylation-specific PCR are well suited for detecting low-abundance metabolites in various biological fluids and DNA in plasma and tissues for biomarker investigation. They offer great clinical promise for early disease diagnosis and therapeutic intervention. This dissertation broadly organized into two parts, part one talks about the application of LC-MS/MS for drug studies and part two converses about cancer diagnostics using PCR.

Part I, The LC-MS/MS quantitative method is essential for the study of pharmacokinetic and toxicological properties in drug screening. Dependent on the type of molecule analyzed, different methods were established to achieve accurate and reliable detection. LC-MS/MS methods were developed and validated for quantitative analysis of an anti-cancer agent *CSUOH0901*, and an anti-parasitic agent *BMCL26*.

Part II, a Panel of methylated DNA biomarkers for early detection of hepatocellular carcinoma (HCC) was developed by using methylation-specific PCR method. We also developed combination biomarkers for HCC screening. This marker combination enabled sensitive and specific detection of DNA hypermethylation on several tumor-associated genes. The studies with FFPE tissue samples successfully differentiated between HCC and normal tissue samples.

TABLE OF CONTENT

	Page
ABSTRACT.....	v
LIST OF TABLES.....	xvii
LIST OF FIGURES.....	xix

PART-I

CHAPTER I: INTRODUCTION OF APPLICATION OF ADVANCED ANALYTICAL TECHNOLOGIES TO SMALL MOLECULES BY CHROMATOGRAPHY AND MASS SPECTROMETRY

1.1. General introduction of bioanalytical methods and their applications	2
1.1.1. Bioanalysis applications in drug discovery and development.....	4
1.2. Modern bioanalytical technologies.....	5
1.2.1. Principals of LC-MS.....	6
1.2.1.1. LC separation.....	6
1.2.1.2. MS detection.....	10
1.2.2. Mobile phase optimization.....	18

1.2.2.1. Sample preparation.....	19
1.2.2.2. HPLC separation.....	20
1.2.2.3. Matrix effect.....	24
1.3. Method validation.....	27
1.4. References.....	33

CHAPTER II: A RAPID AND SENSITIVE LC-MS/MS METHOD FOR QUANTIFICATION OF A NOVEL ANTITUMOR AGENT, IN RAT PLASMA

2.1. Introduction of anti-cancer agent CSUOH0901.....	36
2.2. Experimental.....	40
2.2.1. Chemical and reagents.....	40
2.2.2. Calibration standard and quality control samples.....	40
2.2.2.1. Preparation of stock and working solutions.....	40
2.2.2.2. Preparation of calibration and quality control plasma samples.....	41
2.2.3. Sample extraction.....	41
2.2.4. LC-MS/MS analysis.....	41
2.2.5. Analytical method validation.....	44
2.2.5.1. Calibration curve, linearity, and sensitivity.....	44
2.2.5.2. Accuracy and precision.....	44
2.2.5.3. Recovery and matrix effect.....	45

2.2.5.4. Stability studies.....	45
2.2.5.4.1. Effect of freeze-thaw on CSUOH0901 in plasma.....	45
2.2.5.4.2. Short term and long term stability studies of analyte in plasma.....	46
2.2.5.4.3. Stability of analyte in stock solutions.....	46
2.3. Results and discussion.....	46
2.3.1. Optimization of mass spectrometric conditions for MRM quantification...	46
2.3.2. Optimization of HPLC conditions.....	47
2.3.3. Linearity, sensitivity, selectivity, and LLOQ.....	49
2.3.4. Accuracy and precision.....	51
2.3.5. Extraction recovery and matrix effect.....	53
2.3.6. Stability.....	55
2.4. Conclusion.....	58
2.5. Acknowledgments.....	58
2.6. References.....	59

**CHAPTER III: A SIMPLE AND RAPID LC-MS/MS METHOD FOR THE
DETERMINATION OF BMCL26, A NOVEL ANTI-PARASITIC AGENT, IN
RAT PLASMA**

3.1. Introduction	63
3.2. Experimental.....	66
3.2.1. Chemical and reagents.....	66
3.2.2. Calibration standard and quality-control samples.....	69
3.2.2.1. Preparation of stock and working solutions.....	69
3.2.2.2. Calibration and preparation of quality-control (QC) plasma samples.....	69
3.2.3. Sample extraction.....	69
3.2.4. LC-MS/MS analysis.....	70
3.2.5 Analytical method validation.....	74
3.2.5.1. Linearity and calibration.....	74
3.2.5.2. Accuracy and precision.....	74
3.2.5.3. Extraction recovery and matrix effect.....	75
3.2.5.4. Stability studies.....	75
3.3. Results and discussion.....	76
3.3.1. Optimization of mass spectrometric conditions for quantification.....	76
3.3.2. Optimization of HPLC conditions.....	76

3.3.3. Linearity, sensitivity, selectivity, and LLOQ.....	79
3.3.4. Accuracy and precision.....	81
3.3.5. Extraction recovery and matrix effect.....	83
3.3.6. Stability.....	86
3.4. Conclusion.....	88
3.5. Acknowledgments.....	88
3.6. References.....	89

PART-II

CHAPTER IV: REVIEW OF HCC AND DNA METHYLATION

4.1. Introduction.....	94
4.1.1. DNA methylation and carcinogenesis.....	95
4.1.2. Hypermethylation in cancer.....	95
4.1.2.1. CpG islands.....	96
4.1.2.2. Global hypo methylation in cancer.....	98
4.1.3. DNA methylation machinery.....	98

4.1.4. DNA methyltransferases (DNMTs)...	101
4.1.5. Methyl CpG binding domain proteins.....	102
4.1.6. Histone modification enzymes.....	102
4.2. Hepatocellular carcinoma.....	103
4.2.1. Epidemiology of hepatocellular carcinoma.....	104
4.2.2. Risk factor for hepatocellular carcinoma.....	105
4.2.3. Molecular mechanisms of hepato-carcinogenesis.....	106
4.2.4. Staging and diagnosis of hepatocellular carcinoma.....	108
4.2.5. Current treatment options.....	109
4.2.6. Current trends in research of biomarkers for hepatocellular carcinoma.....	110
4.3. DNA methylation analysis techniques.....	111
4.3.1. Methylation-specific PCR (MSP).....	114
4.3.2. Bisulfite conversion.....	114
4.3.3. Principle of bisulfite conversion.....	115
4.3.4. Primer design.....	118
4.3.5. Applications of MSP.....	118
4.4. Biomarkers.....	119

4.4.1. DNA methylation as a bio-marker.....	120
4.4.2. DNA methylation as a diagnostic marker.....	121
4.4.3. DNA methylation as a prognostic and predictive bio-marker.....	121
4.5. References.....	123

CHAPTER V: QUANTITATIVE METHYLATION ANALYSIS OF TUMOR ASSOCIATED GENES USING METHYLATION SPECIFIC PCR FOR THE DETECTION OF HEPATOCELLULAR CARCINOMA

5.1. Introduction.....	135
5.1.1. MiR-129-2 as a biomarker.....	150
5.1.2. CHFR as a biomarker.....	150
5.1.3. BLMH as a biomarker.....	151
5.1.4. B4GALT1 as a biomarker.....	152
5.1.5. GLOXD1 as a biomarker.....	153
5.2. Materials and methods.....	153
5.2.1. Collection of clinical tissue specimens.....	153
5.2.2. DNA extraction and purification protocol.....	155

5.2.3. Reagent preparation.....	159
5.2.3.1. Preparation of CT-conversion reagent.....	159
5.2.3.2. Preparation of M – wash buffer.....	161
5.2.4. Protocol for bisulfite conversion.....	161
5.2.5. Quantitative methylation-specific PCR.....	163
5.2.6. Specificity and sensitivity of MSP.....	165
5.2.7. Statistical analysis.....	165
5.3. Results.....	165
5.3.1. Specificity and sensitivity of MSP method.....	165
5.3.2. Quantitative methylation analysis.....	168
5.3.3. Gene-specific promoter methylation analysis.....	172
5.3.4. Methylation levels of multigene in HCC.....	176
5.3.5. Correlation with clinicopathologic parameters.....	180
5.4. Discussion.....	180
5.5. Conclusion.....	183
5.6. References.....	184

CHAPTER VI: FUTURE RECOMMENDATIONS

6.1. Pharmacokinetic studies of anti-parasitic drug (BMCL26) in rat models.....	201
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6.1.1. Experimental methods and design.....	202
6.1.1.1. Animals.....	202
6.1.1.2. Number of rats.....	202
6.1.1.3. Drug administration.....	203
6.1.1.4. Multiple blood sampling.....	203
6.1.2. Analysis.....	204
6.1.2.1. Sample extraction.....	204
6.1.2.2. Area under curve.....	205
6.1.2.3. T_{\max}	205
6.1.2.4. C_{\max}	206
6.1.2.5. T half-life.....	206
6.1.2.6. Volume of distribution [Vd].....	206
6.1.2.7. Clearance.....	206
6.1.2.8. Bioavailability [F].....	207
6.2. Development of novel blood DNA test for early detection of hepatocellular carcinoma.....	207
6.2.1. Experimental methods and design.....	208

6.2.1.1.	Extraction of DNA form blood/plasma sample.....	208
6.2.1.2.	Sodium bisulfite conversion.....	209
6.2.1.3.	Methylation analysis.....	209
6.2.1.4.	Statistical analysis.....	210
6.2.1.4.1.	Specificity and sensitivity of biomarkers.....	210
6.2.1.4.2.	Likelihood ratio.....	211
6.2.1.4.3.	Positive predictive value.....	211
6.2.1.4.4.	Negative predictive value.....	212
6.2.1.4.5.	Receiver operator characteristic curves.....	212
6.2.1.5.	Combination of biomarkers.....	212
6.3.	References.....	213

LIST OF TABLES

Table	Page
2.1. Accuracy and precision of CSUOH0901 calibration standards [n=5] over 0.5 – 100 ng/ml.....	50
2.2. Inter and intra-assay accuracy and precision of CSUOH0901 in rat plasma	52
2.3. Absolute and relative matrix effect and recovery of CSUOH0901 in rat plasma.....	54
2.4. Stabilities of CSUOH0901 under various conditions.....	56
2.5. Stabilities of CSUOH0901 and JCC76 stock solutions after storage at -20 °C for 6 months.....	57
3.1. HPLC gradient program.....	72
3.2. Accuracy and precision of BMCL26 calibration standards in rat plasma (n=5, pooled plasma samples).....	80
3.3. Inter and intra-assay accuracy and precision of BMCL26 in rat plasma.....	82
3.4. Absolute and relative matrix effect of BMCL26 in rat plasma.....	84
3.5. Absolute and relative extraction recovery of BMCL26 in rat plasma.....	85
3.6. Stability of BMCL26 in plasma samples.....	87
5.1. DNA hypermethylation genes list for HCC from 2000-01-01 to 2015-07-30.....	138

5.2. Clinicopathological parameters of 80 patients used in the independent validation study by qMSP.....	154
5.3. FFPE tissue DNA samples concentrations.....	158
5.4. CT-conversion reagent.....	160
5.5. Primer sequences for quantitative methylation specific polymerase chain reaction.....	164
5.6. The methylation percentage of 5 genes and combination of 5 genes on 80 pair of HCC cancerous/non-cancerous samples.....	171
5.7. Diagnostic ability of five methylated genes based on qMSP analysis.....	174

LIST OF FIGURES

Figures	Page
1.1. The instrumentation setup for LC separation.....	9
1.2. The schematic diagram of ESI source.....	12
1.3. The operation of quadrupole mass analyzer.....	14
1.4. A schematic way of a ion-trap mass analyzer.....	16
1.5. Method development workflow for small molecules.....	23
2.1. The chemical structures of CSUOH0901 (A), internal standard JCC76 (B), and nimesulide (C).....	38
2.2. Precursor/product ion spectra and proposed fragmentation pathways for internal standard JCC76 (A) and analyte CSUOH0901 (B).....	43
2.3. (A) MRM chromatograms of blank rat plasma in both IS and analyte windows (B) IS JCC76 (10 ng/ml, 5.58 min) and CSUOH0901 at LLOQ level (0.5 ng/ml 5.10 min).....	48
3.1. The chemical structures of JCC76 (A), analyte BMCL26 (B).....	68
3.2. Precursor/product ion spectra and proposed fragmentation pathways for internal standard JCC76 (A) and analyte BMCL26 (B).....	73
3.3. (A) MRM chromatograms of blank rat plasma in both IS and analyte windows (B) IS JCC76 (10 ng/ml, 3.12 min) and BMCL26 at LLOQ level (0.5 ng/ml 2.48 min).....	78

4.1. The methylation state of a gene is equilibrium of methylation and demethylation reaction.....	97
4.2. Epigenetic provides a new generation of oncogene and tumor-suppressor genes...	100
4.3. Molecular mechanisms of hepatocellular carcinoma at the cirrhotic stage.....	107
4.4. DNA methylation analysis techniques.....	113
4.5. Bisulfite mediated conversion of cytosine to uracil.....	116
5.1. (A) Standard β -actin curve. (B) amplification curve for FFPE samples.....	157
5.2. The amplification results of standard methylated DNA 1% methylated DNA, standard unmethylated DNA as negative control, and blank control are shown in RT-PCR (A) and melting curve analysis (B).....	167
5.3. Standard curve constructed for quantifying methylated DNA for gene MiR-129-2 (A) and amplification curve for the MiR-129-2 standard (B).....	170
5.4. Standard curve constructed for quantifying methylated DNA for combination of 5 genes (A) and amplification curve for the combination of 5 genes (B).....	173
5.5. Receiver-operating characteristics (ROC) curve for the combined analysis of DNA methylation levels (GLOXD1, B4GALT1, CHFR, BLMH, and miR-129-2) in 80 paired HCC and adjacent non-tumorous tissues. AUC: area under the curve.....	175

5.6. Quantitative methylation results of qMSP on Hepatocellular Carcinoma and Adjacent non-tumorous tissues. The line represents the cut-off value. Mann-Whitney U test was used to determine statistical significance.....	177
5.7. Quantitative methylation results of qMSP for combined genes on hepatocellular carcinoma and adjacent non-tumorous tissues.....	178
5.8. Receiver-operating characteristics curves for individual gene analysis of DNA methylation levels in discriminating HCC from adjacent non-cancerous tissues.....	179

Part - I

CHAPTER I

INTRODUCTION OF APPLICATION OF ADVANCED ANALYTICAL TECHNOLOGIES TO SMALL MOLECULES BY CHROMATOGRAPHY AND MASS SPECTROMETRY

1.1. General introduction of bioanalytical methods and their applications

Bioanalysis is a term generally used to describe the qualitative and quantitative measurements of a compound or their metabolites in biological materials. A bioanalytical method mainly contains two components i) Sample preparation ii) detection of the compound. Bioanalytical science plays a key role in understanding diseases, clinical diagnosis, and drug discovery and development. The technologies in biomedical science have made significant progress over recent years. This facilitates bioanalytical method development to become an integral component of biomarker discovery, drug

metabolism/pharmacokinetic (DMPK), and toxicological monitoring. Advanced technologies and enhancements of conventional platforms emerged from bioanalysis fulfill the requirements of clinical and pharmaceutical fields, including the improvement in mass spectrometry detection, fast chromatographic separation, high-throughput samples pretreatment, and melting curve analysis with high resolution for genomic assays.

Early diagnosis of diseases has great significance in improving surgical rates and minimizing current invasive diagnostic procedures. This leads to another major clinical need in the accurate detection of molecular biomarkers for chronic illnesses and cancers. The biomarkers study monitors different biological entities including nucleic acids, proteins, and metabolites to reflect the pathophysiology and progression of diseases. Ideal biomarkers need to be well understood for their functions in the pathogenic process and their values for clinical diagnostics, prognostic, and predictive outcomes. However, these molecular biomarkers often present in low abundance in the biological samples, bringing great challenges in reliable detection and validation. In spite of these challenges, there are a large number of biomarkers developed currently that must be validated in clinical studies for their diagnostic and prognostic applications [1].

Besides the broad bioanalytical applications in biomarkers discovery, the impressive growth of quantitative bioanalysis has been also well documented in pharmaceutical drug discovery and development. In the past decade, more than 500 novel drugs were approved by U.S. Food and Drug Administration (FDA) to prevent and treat human diseases [2]. Each year, more than 3000 on-going clinical trials are carried out in the drug development phase [3]. Despite the enormous amount of lead compounds screened in the drug discovery phase, the drug development process is costly and risky with very low rate of clinical

success. This drives the rational lead optimization in the earliest stage of drug discovery to improve the likelihood of drug approval and prevent drug withdrawal on the market. Quantitative bioanalysis serves as a major tool for understanding pharmacological properties including absorption, distribution, metabolism, and elimination (ADME), as well as toxicity to guide the drug screening for lead candidates.

1.1.1. Bioanalysis applications in drug discovery and development

Drug PK and toxicity properties are key parameters in the screening and optimization of lead compounds in the drug discovery phase. An ideal drug candidate should demonstrate the ability be absorbed in the blood stream, reach desirable concentration for effective activity, and be eliminated without producing toxic metabolites.

High-throughput PK screening usually starts from in-vitro assay to study the drug-drug interaction and metabolism using liver microsomes and hepatocytes as experimental systems. However, the in-vitro results cannot truly represent the real physiological environment and may lead to a mistaken conclusion about drug metabolism. Therefore, it is essential to assess the PK parameters in vivo to improve the candidate selection through animal models.

In order to accurately define the drug behaviors in vitro and in vivo, bioanalytical support has been a prerequisite in the pharmaceutical industry. A large amount of compounds involved in the lead optimization requires the quantitative method to be accurate, sensitive, and high-throughput to facilitate drug discovery. These requirements can be fulfilled by accurate sampling procedures, advanced chromatographic and mass spectrometric techniques, as well as automated sample preparation methods.

1.2. Modern bioanalytical technologies

Modern bioanalytical technologies have been significantly broadened in the last decade, demonstrating its ability in accurate qualitative and quantitative determination of protein, nucleic acids, small molecular metabolites, and drug in biological materials. The major methodologies used for proteomics investigation are based on mass spectrometry (MS). The dramatic progress of MS instrumentation refines mass accuracy, resolution, and dynamic ranges, ensuring the successful detection of low abundance proteins in biofluids and structural confirmation with their characteristic precursor and fragment ions. In addition, the robust and reliable liquid chromatography (LC) system in low flow rate has greatly improved the sensitivity for the MS detection and confidence for structure illustration.

Besides its application in protein analysis, the hyphenation of LC and MS (LC-MS) is established as the state-of-the-art methodology for the quantitation of small molecular compounds due to its specificity and sensitivity. It is now widely accepted as the preferred method for the quantitative measurement of small molecule drugs and endogenous metabolites in various biological matrices including plasma, serum, blood, urine, intestinal fluid, and tissue.

With respect to epigenetic biomarker discovery, the majority of DNA methylation assays are based on bisulfite reaction, methylation-specific PCR (MSP), and melting curve analysis. Sodium bisulfite converts cytosine to uracil at unmethylated CpG site, leaving methylated one unchanged. The MSP methods with designed primers selectively amplify methylated DNA bringing high analytical specificity and sensitivity.

1.2.1. Principals of LC-MS

1.2.1.1. LC separation

LC is the basic separation platform for bioanalysis. With this technique, the target analyte can be separated with interfering protein, salts, and phospholipids content in complicated biological materials. The separation mechanisms of liquid chromatography are based on the distribution of analyte between the liquid mobile phase and a stationary phase. Depending on the different type of stationary phases, different distribution mechanisms are applied.

Adsorption mechanism is applied for both normal-phase (NP) chromatography and reverse-phase (RP) chromatography. In NP chromatography, the stationary phase is a polar silica gel and the mobile phase is a non-polar solvent such as hexane, pentane, and chloroform. NP chromatography is preferable for the non-polar analyte and the retention decreases as the non-polarity of analyte increases. Opposite to NP chromatography, the stationary phase of RP chromatography uses non-polar silica-based packing materials after the surface modification with C8, C18, or phenyl. Accordingly, the retention decreases with increasing polarity of the compound and a number of polar solvents. RP chromatography is suitable ideally for polar and ionic compounds, which makes it the most widely used LC application. The interaction of the analyte with stationary phase and mobile phase solvent greatly depends on the hydrophobicity of the analyte.

Ion-exchange chromatography is based on the ion exchange equilibrium between the ionic or polar compounds with the stationary phase. With opposite change with the ionic

functional group of the stationary phase, the ionic compounds can be retained. The elution speed is related to the ionic strength of the counter-ions, p^H environment, and the modifier contained in the mobile phase.

Size-exclusion chromatography is usually applied in the separation of macromolecules according to their ability to penetrate into the pore of stationary material. The elution time of analyte is merely based on their size, but not molecular weight. The retention decreases as the size increases.

Besides the aforementioned traditional chromatography, there are some modern approaches for improving chromatographic resolution and separation efficiency: ultra-performance liquid chromatography (UPLC), monolithic chromatography, and hydrophilic interaction chromatography (HILIC). Underlying the same basic principle with RP-LC, UPLC utilizes column with sub-2 μm particle size and a system that can handle evaluated pressure. UPLC has a great advantage in resolution, sensitivity, and speed over conventional HPLC, and thus is considered as a better tool for high throughput analysis. Monolith column is packed with highly porous material, which is designed to handle fast flow rate and ensure sufficient surface for separation at the mean time. Consequently, the separation speed and sample throughput are significantly increased. HILIC is a valuable alternative to NP chromatography for very polar compounds because polar compounds are hardly retained and experience bad reproducibility when using NP chromatography. In addition, the large portion of organic mobile phase used for the HILIC elution increases the sensitivity when coupling MS with LC for detection.

In general, a sample is separated and analyzed by LC in the following sequence: the sample solution is injected through an injection port, and then delivered by the mobile phase by high-pressure pumps, and finally flowed into the column for retaining and further elution (Fig. 1.1).

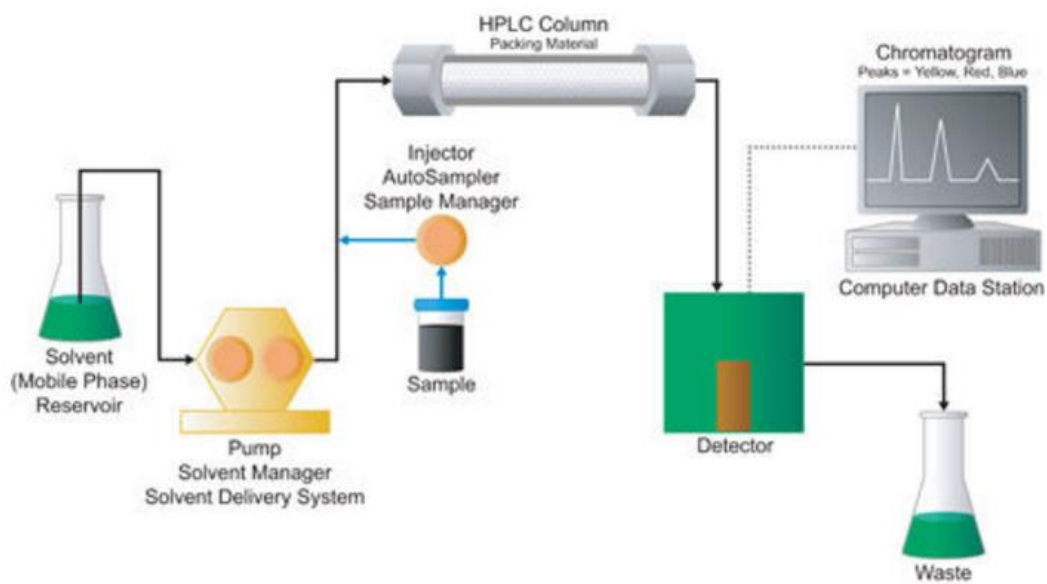


Figure 1.1, The instrumentation setup for LC separation

The instrumentation design should consider the following issues: the high-pressure is generated when the solvents are pumped into the small particle filled stationary phase: the dead volume of connecting tubes, the injector, and the mixing valve should be minimized to prevent the reduction of analyte peak resolution; the sample residue on the tubing and injector should be avoided for carry-over issue in quantitative analysis.

Recent bioanalytical technologies have been considerably expanded over the past decade for quantitative determination of small molecules and large molecules in biological samples accurately. The combination of high-performance liquid chromatography (HPLC) with tandem mass spectrometry (MS/MS) detection is considered as the preferred method for the quantitative determination of drugs and metabolites in biological fluids due to its high sensitivity, selectivity, and short analysis time [9-11]. In the pharmaceutical analysis, the hyphenated LC-MS is the most powerful technique because of the complex nature of the matrix (often plasma or urine) and the need for high sensitivity to observe concentrations after a low dose and a long time period [9, 12].

The most common instrumentation used in this application is [LC-MS](#) with a [triple quadrupole mass spectrometer](#) with ESI or APCI interface. Tandem mass spectrometry is usually employed for added specificity [9].

1.2.1.2. MS detection

The general mechanism of MS detection is the analyte in liquid flow from the HPLC goes into three components of the mass spectrometer of ionization source in which the sample

is ionized, a mass analyzer in which the ions are separated and sorted according to their mass and charge ratio, and the detector which measures and detects the separated ions. The mass spectrometry is capable of not only indicating the presence of impurities but also able to give the molecular weights of underlying components and to distinguish between different molecules.

HPLC/MS became a success with the introduction of atmospheric pressure ionization (API) ten years back that enables the MS analysis by generating ions in a stream of liquid after HPLC separation. The most widespread method for quantifying small molecules is quadrupole MS interfaced with a number of API sources such as electrospray ionization (ESI) or atmospheric pressure chemical ionization (APCI). These ionization sources convert the sample from liquid phase to gaseous state [9].

In the ESI source, a highly positive or negative voltage is applied to the end of a steel capillary probe, where the sample solution is introduced. In electrospray ionization, the analyte is introduced to the ion source in solution either from a syringe pump or as the eluent flow from liquid chromatography. The analyte solution flow passes through the electrospray needle that has a high potential difference (with respect to the counter electrode) applied to it (typically in the range from 2.5 to 4 Kv). This forces the spraying of highly charged fine droplets from the needle with a surface charge of the same polarity to the charge on the needle. After the solvent is further evaporated from these droplets, the droplet shrinks until it reaches the point that the surface tension can no longer sustain the charge at which point a coulombic explosion occurs and the droplet is diffused apart leaving the ions to enter to the mass analyzer. The process of ESI is shown in Fig.1.2.

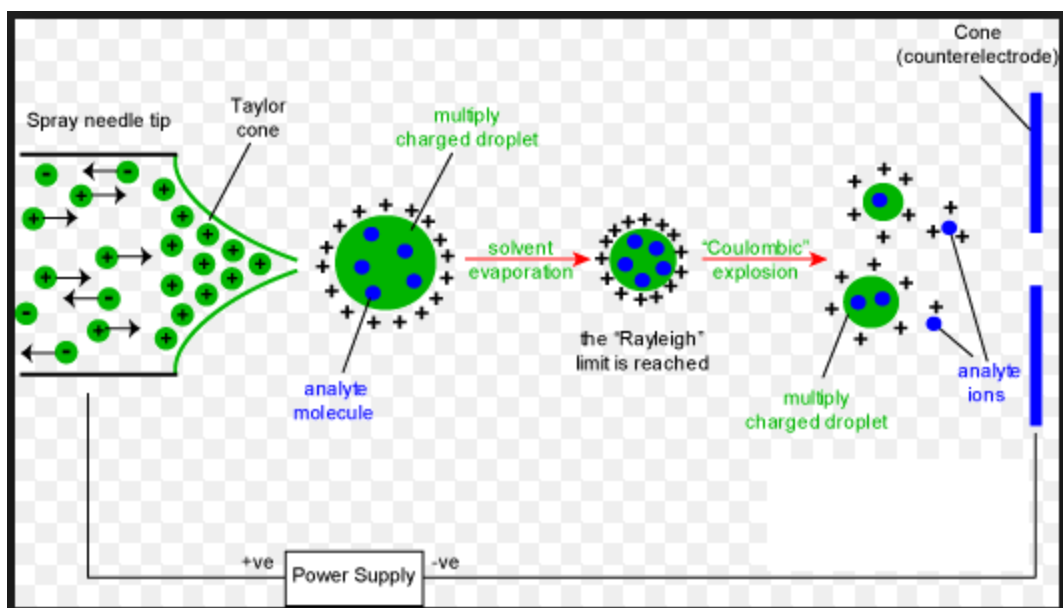


Figure.1.2, The schematic diagram of ESI source

Unlike ESI, APCI evaporates the solvent by passing it through a heated tube where the high voltage is applied to the needle to generate a corona discharge and forming the plasma ions in the solvent. These solvent ions ionize the analyte molecules by means of gas phase reactions[9].

Mass analyzers separate the ions according to their charge to mass ratio (m/z) by accelerating the ions with applying electric or magnetic field. Based on mass range limit, analysis speed, mass accuracy, and resolution, there are different types of mass analyzers are used. These include quadrupole MS, ion-trap MS, and time of flight (TOF) MS.

The quadrupole mass analyzer [Fig.1.3] uses an electric field to select and separate the ions with a particular m/z . It consists of four parallel metal rods where adjacent rods have opposite voltage polarity applied to them. The voltage applied to each rod is the summation of a constant DC voltage and a varying radio frequency.

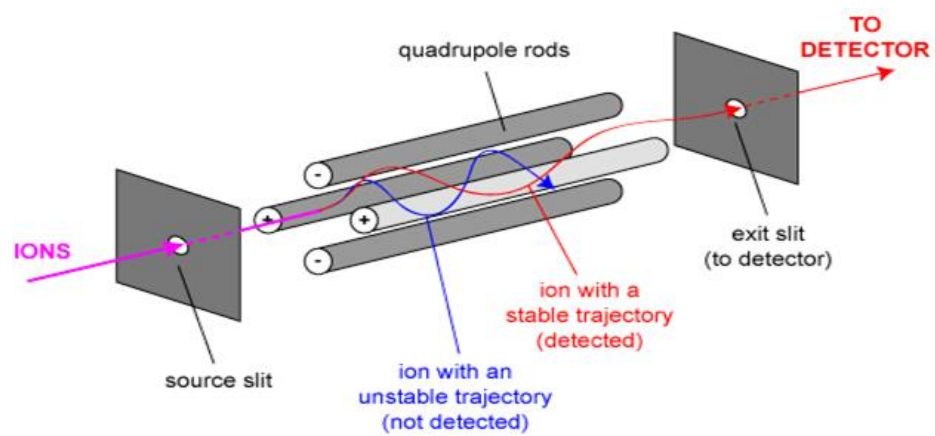


Figure.1.3, The operation of quadrupole mass analyzer

The electric force on the ions causes the ions to travel down the quadrupole between the rods. Only ions of a certain [mass-to-charge ratio](#) will reach the detector for a given ratio of voltages. Quadrupole analyzers have a limited m/z range, high sensitivity, and mass accuracy, but low percentage of ion transmission. Triple quadrupole (QQQ) mass analyzer consists of three quadrupoles arranged in a linear series. Precursor ions filtered in the first quadrupole (Q1) are dissociated in the collision cell (q_2) in the presence of an inert gas such as Ar, He or N₂ gas, and resulting selected fragments are filtered or scanned by third quadrupole (Q3) achieving high specificity.

The quadrupole ion-trap mass analyzer employs similar principles as the quadrupole analyzer mentioned above, it uses an electric and magnetic fields for the separation of the ions by mass to charge ratios. The ion-trap MS (Fig.1.4) has the advantage of its high sensitivity and resolution.

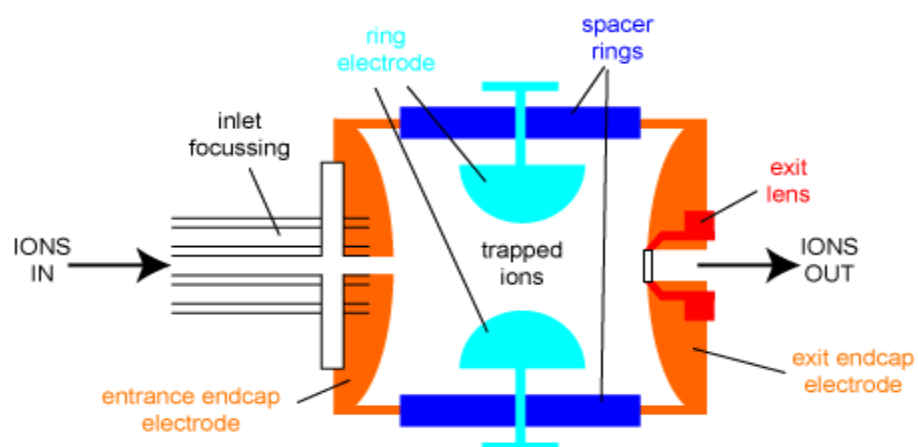


Figure.1.4, A schematic way of an ion-trap mass analyzer

Time-of-flight (TOF) mass spectrometers use an electric field to accelerate gas phase ions toward a detector. The m/z of an ion will determine how long it takes to travel from the source to the detector, with low m/z ions traveling faster relative to high m/z ions. Several designs of TOF analyzers exist, some using a linear flight tube and others using one or more reflectrons that change the direction of ion flight and improve resolution or the ability to distinguish two m/z ratios from one another. TOF analyzers have an essentially unlimited m/z range and very high sensitivity, mass accuracy, and percentage of ion transmission, but a limited dynamic range. The modern MS instrument hybridizes different types of mass analyzers on one instrument to broaden the tandem mass spectrometry applications.

The mass spectrometric detection of target analyte ions employs selected ion monitoring (SIM) and multiple reaction monitoring (MRM) scanning modes for LC-MS and LC-MS/MS methods respectively. The SIM mode detection allows selecting the desired m/z value for the target analyte by the instrument. This mode of analysis requires a single quadrupole and only the m/z of a precursor ion because no fragmentation is induced. Because only a limited [mass-to-charge ratio](#) range is transmitted or detected by the instrument, this operation typically results in significantly increased sensitivity.

Comparing with SIM, MRM mode detection provides the unparalleled specificity because this analysis utilizes triple quadrupole mass analyzer to select and analyze a specific analyte. In MRM mode, two stages of mass filtering are employed on a [triple quadrupole mass spectrometer](#). In the first stage, an ion of interest (the precursor) is preselected in Q1 and induced to fragment by collisional excitation with a neutral gas in a pressurized collision cell (q2). In the second stage, instead of obtaining full scan MS/MS where all the

possible fragment ions derived from the precursor are mass analyzed in Q3, only a small number of sequence-specific fragment ions (transition ions) are mass analyzed in Q3. This targeted MS analysis using MRM enhances the lower detection limit as compared to full scan MS/MS analysis by allowing rapid and continuous monitoring of the specific ions of interest. Particular precursor ions and product ions are selected for detection based on their unique pathways, MRM provides a great improvement in signal to noise ratio.

1.2.2. Mobile phase optimization

Mobile phase additives are often added in RP-LC for reproducible retention and the improvement of resolution and sensitivity when using MS as a detector. However, only volatile additives are compatible with LC-MS because the non-volatile buffers such as phosphates may clog the ionization source and cause signal suppression. In addition, some volatile additives help the retention but deteriorate the MS ionization. For example, trifluoroacetic acid (TFA) is commonly used as an ion-pairing agent for increasing the retention of polar compounds. Nevertheless, it is also reported to induce significant signal suppression for some negatively and positively charged compounds [13-15].

Besides the LC modifiers, the pH of the mobile phase also has a large impact on both retention and ionization. By adding volatile acids as formic acid, acetic acids, and their salts with ammonium in the mobile phase, the protonation of basic molecules under positive ionization mode is favored in acidic condition. Similarly, the deprotonation of some acidic molecules in negative-ion mode can increase the response by adding ammonium hydroxide as mobile phase additive. However, these conditions may cause an

adverse effect on retention if the hydrophobic interaction between analyte ions with the stationary phase is not sufficient [16]. In addition, the concentration of the additives is also critical since the MS response is reduced at high concentrations and lacks buffer capacity at low concentrations. To solve the dilemma between retention and ionization, the selection of mobile phase composition needs a careful consideration of all the characteristics of an individual analyte.

1.2.2.1. Sample preparation

Although LC is a powerful tool for separation, the sample pretreatment for a biological sample before injecting to LC-MS is essential for accurate and reproducible analysis. The biological samples matrices are very complicated with much higher content in plasma salts, and endogenous lipids than target analyte. The large protein content in plasma sample is problematic due to clogging of the column and reducing analytical efficiency. In addition, the endogenous interference and salts in most biological samples may suppress the ionization of analyte.

Conventionally, the sample cleanup has been performed by protein precipitation (PPT), liquid-liquid extraction (LLE), and solid phase extraction (SPE). PPT is popular when handling plasma sample because it is simple and fast. But the major disadvantage for PPT is that residues consisted of salts and endogenous materials after the removal of proteins, which may greatly affect the MS detection. Besides the use of organic solvents for denaturing proteins, other PPT additives such as acids, metal ions, and salts were reported to improve the efficiency of protein removal and disrupt the protein-drug binding.

LLE is an efficient technique to separate analyte from sample matrices based on the different distribution in the water-immiscible organic phase and aqueous phase. It is successful in giving excellent sample cleanup. But the disadvantage for LLE include the relatively large sample and solvent consumption, possible formation of emulsion, and unsuitability for hydrophilic compounds. Based on the conventional LLE, the salting-out-assisted-LLE is developed as a more convenient alternative by adding concentrated ammonium salt solution into a mixture of biological sample and water-miscible solvents. In this way, high-throughput LLE can be applied through the automation of the handling process in 96-well plate.

The separation process of SPE method prior to sample analysis is similar to LC separation. The analyte is isolated relying on its affinity difference with the liquid sample solution and the solid SPE sorbents. Depending on the interaction of analyte and the selected solid phase, the SPE sorbents vary from polymer based ion-exchange materials to silica based materials. Typical SPE procedure start with the conditioning of the cartridge by a solvent or water. Then the sample is added onto the cartridge and the analyte interacting with the sorbent is retained. While the interferences are removed after rinsing the cartridge with buffer or solvent, the analyte can be eluted with an organic solvent and further concentrated by evaporation and re-constitution.

1.2.2.2. HPLC separation

Liquid chromatography is the basic separation technique for the separation of the analyte of interest from the components of the mixture. High-performance liquid chromatography

(HPLC) is the most widely used separation method in bioanalysis. The mechanisms of liquid chromatography are based on distribution and partition of analyte between liquid mobile phase and stationary phase. There are different types of chromatographic methods based on different types of stationary phases applied. These include ion-exchange chromatography (IEC), reversed-phase chromatography (RPLC), normal phase chromatography (NPLC), and size-exclusion chromatography (SEC) etc.

Besides traditional HPLC, there are some modern approaches such as ultra-performance chromatography and hydrophilic interaction chromatography (HILIC) to improve the chromatographic separation efficiency and resolution. By using smaller particles, speed and peak capacity can be extended to new limits, termed Ultra Performance Liquid Chromatography (UPLC). This technique takes full advantage of chromatographic principles for separations using columns packed with smaller particles and higher flow rates for increased speed, with superior resolution and sensitivity over conventional HPLC by handling elevated pressure.

All small molecule compounds and peptides are separated by RP-LC with a C18 column and mass spectrometric detection. A guard column is used to prevent the contamination from the injected biological samples and damage from the mobile phase additives. A successful quantitative LC-MS/MS method development requires three important interlinked methodologies: MS detection, chromatographic separation, and sample preparation. The steps involve in general method development process shown in Fig.1.5.

Several types of sample preparation methods are existed to extract the analyte from the biological matrices such as solid phase extraction (SPE), liquid-liquid extraction (LLE), and protein precipitation (PP).

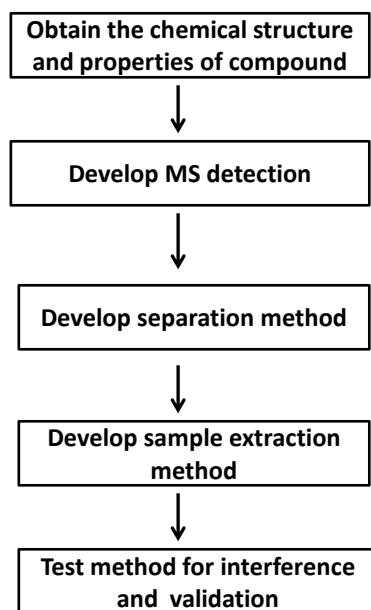


Figure.1.5, Method development workflow for small molecules

1.2.2.3. Matrix effect

Matrix effect is one of the major issues encountered during LC-MS method development and validation. The phenomenon of matrix effect is observed when the ionization of analyte is suppressed or enhanced by the undetected co-eluting components from the biological matrix. The adverse results of matrix effect are reduced sensitivity for the detection and deteriorated precision and accuracy of the assay. According to the FDA guideline, it is required to assess the matrix effect when developing a reliable bioanalytical method.

In order to quantitatively determine the absolute matrix effect, a useful strategy was proposed by matuszewski et al. [17]. The matrix effect is evaluated by comparing the signal response of analyte obtained from a neat solution with that from a post-extraction solution, in this way, two sets of samples are examined: one set is prepared by spiking standard analyte in neat solution and the other set is prepared by spiking standard analyte at the same concentration in the extracted solution of blank biological samples, which is termed as post-extraction solution. The difference of response from these two sets determines whether signal is suppressed or enhanced. More importantly, the relative matrix effect should be evaluated by comparing the response of analyte in post-extraction solution from different blank matrix sources.

The post-column infusion of analyte is usually helpful to locate the co-eluting substances causing suppression in an LC run. A mixing tee is setup after the column elution and prior to MS ionization interface. The post-extraction of a blank biological matrix is injected into the LC system, and then eluted by the mobile phase from the column. At the matrix eluents mix with the analyte, which is infused constantly through an individual syringe pump. The

MS monitors the signal changes after the injection of post-extraction solution. The signal response of analyte should be expected to be steady in the absence of suppressing impurities. When the ion suppression or enhancement of analyte is present, the signal response will drop or increase at certain time points when interferences are eluted out, which can be easily observed on the chromatogram. In this way, the elution time of the ionization interferences and the extent of suppression or enhancement effect can be assessed through several continuous runs. The subsequent experimental design of analyte elution should avoid the co-elution with interferences.

Matrix suppression is induced by different reasons ranged from endogenous compounds from inadequate sample clean-up, ion-suppression mobile phase additives, choice of ionization method, to sample storage conditions. One of the most extensively used and efficient method to solve matrix effect issue is the utility of stable isotope labeled (SIL) internal standard. Since the SIL internal standard has very similar chemical structure and properties compound to the analyte, the ionization suppression or enhancement effect on both compounds is expected to be the same level. However, the SIL internal standard is costly and sometime hard to obtain. It is also problematic for tracking the “cross talk” problem if the purity of SIL internal standard is not adequate.

The matrix effect can also be minimized by improving the sample extraction method to remove the interferences. The endogenous compounds in biological samples have different polarity and thus are difficult to be completely removed by sample extraction methods. However, choosing the optimal sample preparation to reduce the amount of interferences is an efficient approach to ensure success in method development. Little et al. identified the phospholipids as a major contributor of matrix effect in blood and plasma by MS/MS

using different extraction methods [18]. Their results suggested that the glycerophosphocholines caused matrix effect in both positive and negative ionization, with lower effect for isocratic elution than gradient elution. As the effect of different sample pretreatment methods on matrix effect, it is reported that LLE had lower signal suppression compared to SPE, followed by the PPT extract, which usually contains the most endogenous residues.

Alternatively, adjusting the chromatographic conditions is another approach to reduce or eliminate the matrix suppression. In the RP-LC separation, the suppression effect is often found in an early time of isocratic elution program and at the end of gradient elution during the post-column infusion. Therefore, it is wise to alter the elution of target analyte at other regions of the chromatogram where the matrix effect is the lowest. Some mobile phase additives such as trimethylamine (TEA) and TFA can also induce matrix effect in LC-MS analysis. The strong ion-pairing ability of these additives helps trap very polar compounds in the column and reduce peak tailing, but it also masks the detection by neutralization the positive charge of analyte. The use of ammonium salts as a substitution or the choice of other column with different retention mechanism can relieve this problem.

In bioanalytical method development, matrix effect is more frequently reported in ESI interface MS than APCI since the ionization mechanisms are different in these two sources. In ESI, the analyte is charged when traveling in the electrical probe, then nebulized to small droplets, and at last evaporated in the gas phase. When the interfering compounds compete with the analyte for the surface charge, the charge transfer occurs if the interferences have higher proton affinity, causing the loss of charge for the analyte and the decrease of MS intensity. Compared to ESI, the APCI of analyte in liquid undergoes opposite sequence

for evaporation and ionization. The evaporation of liquid solvent takes place in the capillary before the ionization by charge transfer from the corona probe.

In addition to the endogenous compounds causing matrix effect problems, other often neglected sources from the dosing vehicles and blood anticoagulant can also result in ion suppression. Dosing vehicles including propylene glycol, Tween 80, and hydroxypropyl- β -cyclodextrin are often used in the pre-clinical PK studies. Undetected matrix effect in the post-dose samples would give underestimated drug concentration and generate PK results with large errors. It also has been suggested that heparin should be avoided for separating plasma from blood during sample handling. Sodium EDTA usually is preferred for anticoagulation in the PK and toxicokinetics studies for the prevention of matrix effect.

1.3. Method validation

The objective of any analytical method is to obtain an accurate, consistent and reliable measurement of the analyte. It is necessary to evaluate the performance of any analytical method once it has been developed. Method validation is used to judge the quality, reliability and consistency of the results obtained by an analytical method. Validation of analytical methods is required by most regulations and is considered a good analytical practice. Although there are several standards for method validation, FDA (U.S. Food and drug administration) and NTP (National Toxicology Program) guidelines are the most commonly accepted standards for method validation. According to these guidelines, there are several parameters that have to be considered when validating a bio-analytical method.

These parameters include sensitivity, selectivity, matrix factor, recovery, calibration curve, accuracy, precision, and stability. These parameters are described below in details.

Selectivity is the ability of a method to differentiate and quantify an analyte in the presence of other components that may be present in the matrix. typical interfering substances in a biological matrix may include metabolites, impurities, detergents, decomposition products, xenobiotics and other endogenous matrix components.

for non-MS analysis, analyses of blank samples from at least six sources of the matrix must be performed. for MS analysis, if non-isotopically labeled IS is used, matrix factor in six sources should be tested. in the case of an isotopically labeled is, matrix factor of an isotopically labeled IS should be close to unity.

Sensitivity (lower limit of quantification, LLOQ) of an analytical procedure is the lowest amount of analyte in a sample that can be quantitatively measured with suitable accuracy and precision.

The sensitivity of a method should be tested by analyzing at least 5 replicates of the sample at the LLOQ concentration on at least one of the validation days where the accuracy and precision should be lesser than or equal to 20%.

Matrix factor is a measure of the effectiveness of the extraction, fractionation and GC analysis methods. the presence of a matrix effect may result in the ionization enhancement or suppression of analyte of interest.

$$\text{Matrix factor} = (\text{response in matrix}) / (\text{response in pure solution}) \times 100\%$$

Matrix factor is calculated by comparing the ratio of the signals obtained by spiking the standards in the post-extraction matrix to that of the signals obtained in pure solution. to determine the matrix effect, five replicates of QC samples should be determined at three concentration levels (i.e. low, medium and high) of the calibration range.

Recovery of a method is an evaluation of the effectiveness of the extraction methods. it is reported as the percentage of the analyte

$$\text{Recovery} = (\text{response of analyte in spiked analyte}) / (\text{response of analyte in extraction matrix}) \times 100$$

Recovery is calculated by comparing the ratio of the signals obtained by spiking the analyte in the extracted matrix sample to that of the signals obtained by spiking the analyte in the post-extraction blank matrix. To determine recovery, five replicates of QC samples should be determined at three concentration levels (i.e. low, medium and high) of the calibration range.

Calibration standards should be prepared in the same matrix as that of the real samples. In only some special cases where it is impossible to obtain a surrogate matrix or heavy isotope standards, calibration standards may be prepared in a solution matrix if and only if the matrix effect has been proven to be negligible. A calibration curve should include six to eight non-zero standards, one single blank (sample with internal standard] and one double blank [sample without internal standard).

Accuracy of an analytical method is the closeness of the test results obtained to that of the true value of the analyte. The accuracy of a sample can be analyzed by measuring five replicates of the QC samples at three concentration levels (i.e. low, medium and high) in

the range of the calibration curve. The mean values of these measurements should be within 15% of the actual value. Accuracy is expressed as percentage relative error (%RE) and is calculated by the following equation,

$$\%RE = [(measured - nominal)/nominal] \times 100\%$$

Precision of an analytical method is the closeness of the measurements of the analyte obtained by multiple sampling of the same sample under the prescribed conditions. Precision of a sample can be analyzed by measuring five replicates of the QC samples at three concentration levels (i.e. low, medium and high) in the range of the calibration curve. The mean values of these measurements should be within 15% of the actual value. precision is expressed as percentage coefficient of variation (%CV) and is calculated by the following equation,

$$\%CV = (standard\ deviation/mean) \times 100\%$$

accuracy and precision are further subdivided into intra and inter-run accuracy and precision. Intra-run accuracy and precision may be determined by measuring five parallels of the analyte (QC samples) on one of the validation days. these QC samples should include at least three concentration levels (low, medium, and high).

Inter-run accuracy and precision may be determined by measuring five parallels of the analyte (QC samples) on five different validation days. these QC samples should include at least three concentration levels (low, medium, and high).

Stability of an analyte is a measure of the bias in results obtained during a pre-selected time interval that can range from few hours to months. These studies provide important information on sample handling and storage. The most common stability studies include,

stock solution stability, freeze-thaw stability, post-preparative stability, short-term stability and long-term stability. For each stability study, at least two concentration levels (i.e. low and high) should be measured. at each concentration level, five parallel replicates should be determined. The signals of the analyte in the samples after incubation will be compared with those of the freshly made samples. The stability results are expressed as recovery of the analyte, and these indicate the degradation that can be seen by the signal loss.

Stock, solution stability studies, should include a minimum of six hours' study at room temperature. For each study at least two concentrations of the analyte should be studied.

Bench top stability should be performed at ambient temperature conditions or at the temperature used for processing the samples as to cover the duration of time taken to extract the samples. This would typically include the time between 6 to 24 hrs.

Post preparative stability is performed on the samples after they are processed and kept in an autosampler waiting for analysis. The study time should include a minimum time as to cover the total running time of a whole batch of samples.

Freeze and thaw stability involves testing the analyte stability after three freeze and thaw cycles. The study should include at least five replicates at each of the low and high concentrations. for each cycle, samples should be stored and frozen to the intended storage temperature (i.e. -20 °c or -80 °c) for 12-24 hours and thawed unassisted to room temperature.

Short-term stability studies are carried out at room temperature. Five aliquots each of the low and high QC concentrations are spiked into the biological matrix and left on the bench top and kept at this temperature from 4 to 24 hours and analyzed.

Long term stability studies are carried out at a desired storage place such as a refrigerator (4 °c) or in a freezer (-20 or -80 °c). In long-term stability studies, the storage time should exceed the time between the date of first sample collection and the date of the last sample analysis. It is determined by storing five aliquots of each of the low and high concentrations under the same conditions as the study samples.

1.4. References

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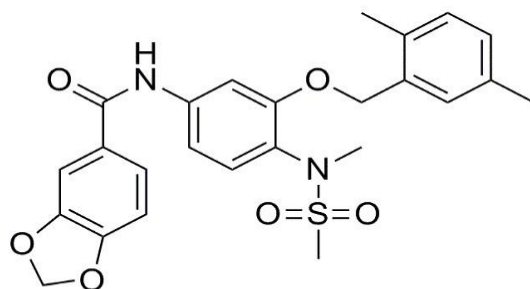
CHAPTER II

A RAPID AND SENSITIVE LC-MS/MS METHOD FOR QUANTIFICATION OF A NOVEL ANTITUMOR AGENT, IN RAT PLASMA

2.1. Introduction of anti-cancer agent CSUOH0901

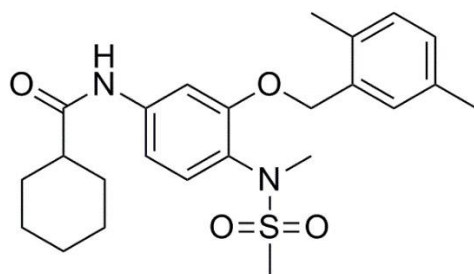
CSUOH0901 {benzo [1, 3] dioxole-5-carboxylic acid [3-(2, 5-dimethylbenzyloxy)-4-(methanesulfonylmethylamino)-phenyl] amide} (NSC751382) (Fig. 2.1A) [1] is a novel, second generation anti-cancer agent derived from Nimesulide, which can inhibit cyclooxygenase-2 (COX-2) (Fig. 2.1C). In cancer therapy, Nimesulide showed hepatotoxicity on long term usage and required higher concentrations to inhibit COX-2 activity [2]. This led to the development of CSUOH0901, a Nimesulide derivative, which exhibited very promising anticancer activities by interacting with tubulin and Hsp27 proteins, which are important to cancer cell proliferation. It was evident that CSUOH0901 inhibited the proliferation of cancer cells of lung, breast, colon, CNS, ovary, renal and

prostate cancer with IC₅₀ of 0.1-0.5 μ M, which is 10 fold more active than JCC76 {N- (3-(2,5-dimethylbenzyloxy)-4-(methylmethylsulfonamido) phenyl) cyclohexanecaboxamide} (Fig. 2.1B) [3] and 1000 fold more potent than nimesulide [1].



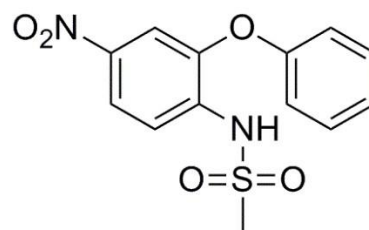
CSUOH0901

[A]



JCC76

[B]



Nimesulide

[C]

Figure. 2.1, The chemical structures of CSUOH0901 (A), internal standard JCC76 (B), and nimesulide (C).

Recent docking studies in SKBR-3 breast cancer cell lines [3, 4] revealed that CSUOH0901 interacted with both α -tubulin and β -tubulin in the colchicine pocket and disorganized microtubules. Additionally, interaction of heat shock protein 27 (Hsp27) [5] with CSUOH0901 inhibited the phosphorylation of Hsp27, leading to cell apoptosis. Hsp27 is a stress protein that is expressed when cells are stimulated by heat [6-8], radiation [9], chemotherapeutic drugs [10], or other agents [11]. A recent study showed that cancer cells with HSP27 overexpression were resistant to chemotherapeutic drugs [12-15]. Antisense to inhibition of the HSP27 gene decreased cellular resistance to chemotherapy as well as to heat shock [16]. Other studies have suggested that HSP27 prevents cancer cells from apoptosis and dramatically enhances their tumorigenicity [17-20]. Mass spectrometric studies revealed that tubulin and Hsp27 proteins are the most prevalent targets of CSUOH0901. Recent in vivo studies demonstrated that CSUOH0901 significantly decreased the size of the HT29 tumor in a Xenograft model compared to the control group, suggesting the low toxicity and high potency in vivo [2].

Clearly, CSUOH0901 is a very promising anticancer drug candidate and will be further studied. However, to date, no LC-MS/MS method has been developed for the quantification of CSUOH0901. Therefore, a simple and accurate method to quantify CSUOH0901 is needed which will be essential to the future pharmacological and toxicological studies of CSUOH0901. In this work, a rapid and sensitive LC-MS/MS method was developed and validated for quantitative determination of CSUOH0901 in rat plasma. We demonstrated that the method developed was fast, sensitive, and specific for quantifying CSUOH0901 in plasma which can be used in pharmacological studies.

2.2. Experimental

2.2.1. Chemical and reagents

CSUOH0901 and JCC76 (Internal standard) were synthesized and purified according to the previously published procedures [1, 3]. HPLC grade methanol and acetonitrile were purchased from Pharmco-Apex (Philadelphia, Pennsylvania, USA). Formic acid, Ammonium formate, and ammonium acetate were purchased from Sigma-Aldrich Chemical Company (Allentown, Pennsylvania, USA). Dimethyl sulfoxide was obtained from Fisher Scientific (Pittsburgh, Pennsylvania, USA). Deionized water was generated from Barnstead Nano pure water purification system from Thermo Scientific (Waltham, Massachusetts, USA). Sprague-Dawley rat plasmas K2 with specific lot numbers (10577-01, 02, 03, 04, 05, and 06) were purchased from Innovative research (Novi, Michigan, USA).

2.2.2. Calibration standard and quality control samples

2.2.2.1. Preparation of stock and working solutions

The stock solutions of CSUOH0901 and JCC76 (IS) were prepared in dimethyl sulfoxide (DMSO) at 1 mg/ml and stored at -20 °C. A set of CSOH0901 working solutions of 10, 20, 50, 150, 400, 1000, and 2000 ng/ml were prepared by serial dilution of the stock solution with DMSO. The working solution of JCC76 (IS) was obtained by diluting the stock solution with DMSO to give a concentration of 150 ng/ml.

2.2.2.2. Preparation of calibration and quality control plasma samples

The calibration plasma solutions were prepared by spiking 10 µl of CSUOH0901 working solutions in 200 µl of blank plasma (mixture of 6 lots) to give drug concentrations of 0.5, 1.0, 2.5, 7.5, 20, 50, and 100 ng/ml. The LLOQ and QC standards were prepared in a similar way at 0.5, 1.25, 10, 80 ng/ml, representing the lower limit of quantification (LLOQ), low QC (LQC), middle QC (MQC), and high QC (HQC) respectively. The QC and calibration samples were frozen at -20 °C overnight, and then treated by the following sample preparation procedure and subjected to LC-MS/MS analysis.

2.2.3. Sample extraction

Plasma samples were removed from -20 °C freezer, and thawed to room temperature. Single and double blanks were prepared by spiking 10 µl of acetonitrile in 200 µl of rat plasma. Then 10 µl of IS working solution was spiked in all calibration, QC solutions and single blank, except in double blank and vortexed immediately for 30 sec. The above-prepared samples were deproteinized by adding 800 µl of acetonitrile and sonicated for 15 minutes followed by centrifugation at $13,000 \times g$ for 15 minutes. The supernatants were transferred into auto sampler vials for LC-MS/MS analysis.

2.2.4. LC-MS/MS analysis

LC-MS/MS analysis was conducted using 5500 QTRAP triple quadrupole, tandem mass spectrometer (AB Sciex, Toronto, Canada) with electrospray ionization (ESI) source

(Framingham, Massachusetts, USA) interfaced with high performance liquid chromatography (HPLC, Shimadzu, Columbia, Maryland, USA) with two LC-30 AD pumps, DUG-20A_{3R} inline degasser, a SIL-30 AC auto sampler, CBM-20A controller and CTO-10AVP column oven (Shimadzu, Tokyo, Japan). Analyst software, version 1.5.2 (AB Sciex) was used to control all the parameters of tandem mass spectrometer and HPLC.

A Luna C18 [2] HPLC column (50×2.0 mm 5 micron) with a C18 security guard cartridge from Phenomenex (Torrance, California, USA) was used for the chromatographic separation of the supernatants from the deproteinized samples. An optimized gradient flow of mobile phase A: 5mM ammonium formate in 2% Methanol and mobile phase B: 5mM ammonium formate in 90% Methanol at a flow rate of 0.2 ml/min was developed. The column was equilibrated with the mobile phase for 10 min and the run time was 8 minutes for each run with 10 μ l injection volume. The positive electrospray ionization (ESI) mode was selected and the MRM (multiple reaction monitoring) function was used for quantification, with the transitions set at m/z 483.2 \rightarrow 404.3 for CSUOH0901 and m/z 445.3 \rightarrow 366.3 for JCC76 (IS) (Fig. 2.2) respectively. The dwell time for each MRM transition was set at 120 ms. Source dependent parameters were optimized by flow infusion analysis: Nebulization gas [30], heating gas [30], curtain gas [40], ion spray voltage [5000ev] and temperature [450°C]. Compound dependent parameters were manually optimized as following: Declustering potential [180], Entrance potential [10], Collision Energy [20], Cell Exit Potential [12].

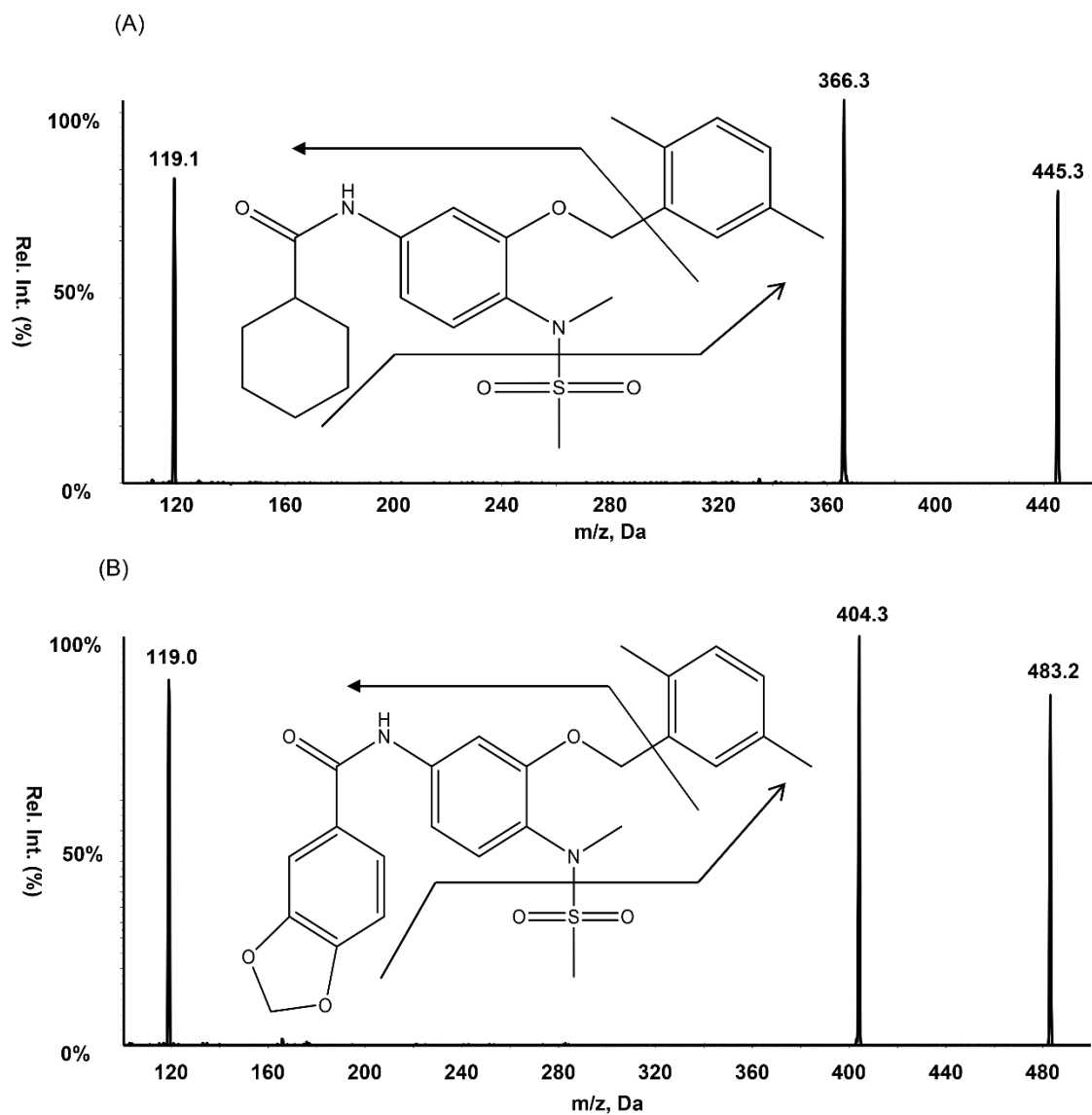


Figure 2.2, Precursor/product ion spectra and proposed fragmentation pathways for internal standard JCC76 (A) and analyte CSUOH0901 (B).

2.2.5. Analytical method validation

A full method validation was performed using rat plasma according to the currently accepted FDA Bio analytical method guidelines [21] and also other references [22, 23]. The entire method was validated for precision, accuracy, linearity, selectivity, extraction recovery, the lower limit of quantification (LLOQ), matrix effect and stability studies.

2.2.5.1. Calibration curve, linearity and sensitivity

Seven CSUOH0901 plasma calibrators at the concentrations of 0.5, 1.0, 2.5, 7.5, 20, 50, 100 ng/ml, double blank and single blank (only JCC76 internal standard) were selected to establish a calibration curve. The weighed linear regression, 1/x as weighing factor was used to calculate the slope and correlation coefficient of the calibration curve. The LLOQ was defined as the concentration with precision (%CV) < 20%.

2.2.5.2. Accuracy and precision

Intra-assay and inter-assay precision and accuracy studies were performed using three QC standards LQC, MQC, and HQC at 1.25, 10 and 80 ng/ml with five replicates (n = 5). Intra-assay and inter-assay precisions determined as % coefficient variance (%CV), and accuracies were calculated by comparing experimentally determined concentrations with the spiked values. Therefore, accuracy (%) = (experimental concentration) / (spiked concentration) x 100.

2.2.5.3 Recovery and matrix effect

The absolute extraction recovery was determined by comparing the peak areas of CSUOH0901 in QC samples at 1.25, 10, and 80 ng/ml (CSUOH0901 added prior to deproteinization), to those of post extraction samples (CSUOH0901 added after deproteinization) of corresponding concentrations. The relative recovery was determined by comparing peak area ratio of CSUOH0901 and IS (JCC76) spiked in plasma before extraction with that in post extraction spiked samples.

The absolute matrix effect was calculated by comparing the peak areas of post extraction blank plasma samples spiked with CSUOH0901 (1.25, 10, and 80 ng/ml) to those of corresponding standard solutions at equivalent concentrations. The relative matrix effect was calculated by comparing the peak area ratio of CSUOH0901 and IS (JCC76) spiked in the blank plasma post extraction solution with that in the standard solution.

2.2.5.4. Stability studies

2.2.5.4.1. Effect of freeze-thaw on CSUOH0901 in plasma

Two QC samples of 1.25 and 80 ng/ml concentrations were selected to verify their stability. Stability test for CSUOH0901 in plasma was studied after three freeze thaw cycles over a three-day period.

2.2.5.4.2. Short term and long term stability studies of analyte in plasma

The Stability studies of CSUOH0901 in rat plasma were performed using two QC standards (1.25 and 80 ng/ml), which were kept under different storage conditions: 10 hours at room temperature and 6 months at -20 °C, before and after sample extraction.

2.2.5.4.3. Stability of analyte in stock solutions

The Stability studies of stock solutions and working solutions of CSUOH0901 and internal standard (JCC76) were also evaluated. The stock solutions of analyte were stored at -20 °C for 7 months. Two QC standards of concentrations 1.25 and 80 ng/ml were prepared from both the stored and fresh stock solutions and the experimentally determined concentrations of CSUOH0901 were compared (n=3 for each sample).

2.3. Results and discussion

2.3.1. Optimization of mass spectrometric conditions for MRM quantitation

Positive ionization mode was selected to detect and optimize the MS parameters for the detection of both CSUOH0901 and JCC76 (internal standard). It was found that the standard CSUOH0901 and JCC76 solutions prepared in methanol-water (9:1, v/v) yielded higher intensity when compared to the solutions prepared in acetonitrile-water (9:1, v/v). Fragmentation lead to the formation of daughter ions in the product ion scan mode (Fig. 2.2). Based on the fragmentation study, the MRM transitions of m/z 483.2 \rightarrow 404.3 for CSUOH0901 and 445.3 \rightarrow 366.3 for JCC76 were selected for quantification, as these

product ions yielded strong signals. The highest MS signal was obtained by fine-tuning collision energy, spray voltage, and ion source temperature.

2.3.2. Optimization of HPLC conditions

To overcome the irreproducibility and matrix effect problems associated with the isocratic flow, a gradient flow of mobile phase A: 5mM ammonium formate in 2% Methanol and mobile phase B: 5mM ammonium formate in 90% Methanol with 0.2 ml/min flow rate was employed. This gradient flow improved the sensitivity and signal-to-noise ratio with a total run time of 18 minutes. High concentration of methanol was used to elute CSUOH0901 from C18 column, due to its low solubility in water with predicted logD value of 4.86. The intensity of CSUOH0901 has increased two folds when 5mM ammonium formate buffer was used in the mobile phases and the retention times were found to be around 5.09 min for CSUOH0901 and 5.58 min for JCC76 (IS) (Fig. 2.3B).

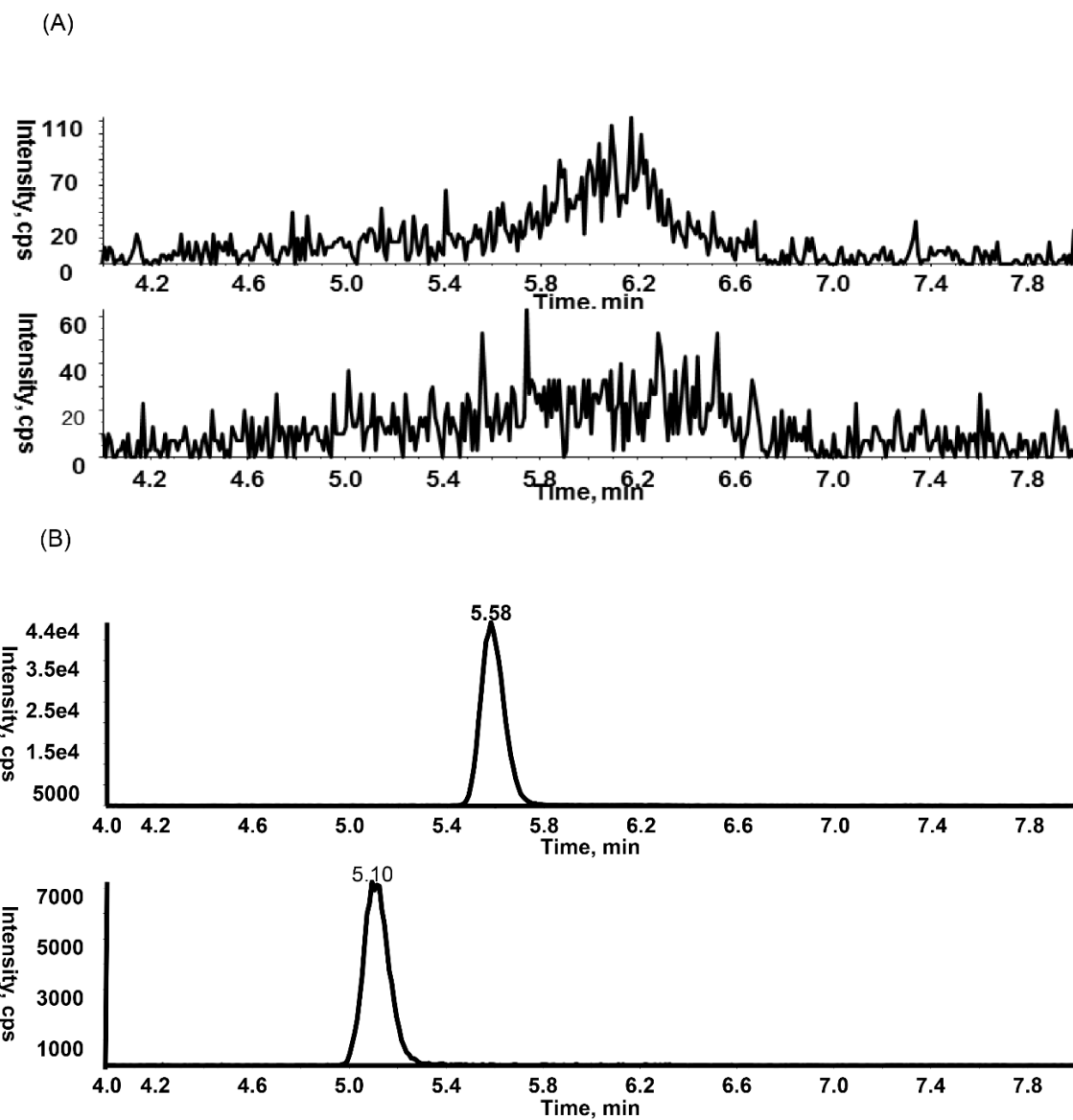


Figure 2.3, [A] MRM chromatograms of blank rat plasma in both IS and analyte windows [B] IS JCC76 (10 ng/ml, 5.58 min) and CSUOH0901 at LLOQ level (0.5 ng/ml 5.10 min).

2.3.3. Linearity, sensitivity, selectivity and LLOQ

The Calibration curve for CSUOH0901 in plasma was linear in the range of 0.5 – 100 ng/ml. Linearity results showed the quadratic fit for CSUOH0901 with a seven-point calibration curve of concentrations 0.5, 1.0, 2.5, 7.5, 20, 50, and 100 ng/ml including double blank and single blank (only JCC76 internal standard) plasma samples. An excellent linearity was obtained with the correlation coefficient of 0.9996 and the linear regression equation was $y = 0.073x - 0.0085$. This method exhibited high selectivity with no interfering peak in six different blank plasma samples from different sources. The LLOQ was found to be 0.5 ng/ml, where the signal intensity was twenty folds higher than the blank signal (Fig. 2.3). The lowest concentration in a calibration curve (LLOQ) was quantified with the accuracy and precision within 15% (Table 2.1).

Table 2.1, Accuracy and precision of CSUOH0901 calibration standards [n=5] over 0.5 – 100 ng/ml

Nominal Concentration	Determined Concentration	Accuracy	Precision
[ng/ml]	[ng/ml]	[%RE]	[%CV]
0.5	0.49±0.01	-2.0%	4.5%
1	0.90±0.09	-10.0%	6.0%
2.5	2.58±0.08	3.2%	6.7%
7.5	7.23±0.27	-3.6%	11.0%
20	20.42±0.42	2.1%	1.7%
50	49.14±0.86	-1.7%	1.6%
100	97.30±2.70	-2.7%	2.2%

2.3.4. Accuracy and precision

Intra-assay and inter -assay accuracies of the validated method ranged from 1.9 to 11.2% and 0.5 to 11.2%, respectively. The Intra-assay and inter –assay precision values ranged from 1.8 to 2.9% and 4.5 to 5.5%, respectively. The inter and intra-assay accuracy and precision of the QC samples are depicted in Table 2.2.

Table 2.2, Inter and intra-assay accuracy and precision of CSUOH0901 in rat plasma

Intra- assay					Inter- assay			
Normal	Determined	Accuracy	SD	Precision	Determined	Accuracy	SD	Precision
[ng/ml]	[ng/ml]	[%RE]		[%CV]	[ng/ml]	[%RE]		[%CV]
1.25	1.39	11.2%	0.04	2.9%	1.39	11.2%	0.07	5.0%
10	10.26	2.6%	0.18	1.8%	10.35	3.5%	0.46	4.5%
80	78.52	-1.9%	1.44	1.8%	79.58	-0.5%	4.36	5.5%

2.3.5. Extraction recovery and matrix effect

The absolute recovery of the extraction method was found to be (104.0%, 105.0%, and 104.0%) of the QC standards at 1.25, 10, and 80 ng/ml, and the relative recovery of the extracted method was found to be (99.9%, 96.1% 97.7%) of the QC standards at 1.25, 10, and 80 ng/ml, respectively as indicated in Table 2.3. Absolute matrix effect for each other plasma samples at 1.25, 10, and 80 ng/ml was 5.5%, 8.1%, and 9.9%, and relative matrix effect was 2.6%, 5.1% and 12.4% indicating the minimal matrix effect. Hence, the protein precipitation technique for sample preparation was found to be effective, as it not only extracted the analyte and internal standard well but also removed impurities causing interferences from the sample matrix.

Performing multiple reaction monitoring (MRM) confirmed the absence of significant matrix effect by comparing the peak areas ratio of CSUOH0901 MRM transitions (MRM₁: m/z 483.2 → 404.3 and MRM₂: m/z 483.2 → 119.0) for the spiked rat plasma samples with the average peak area ratio for seven calibrators.

$$\text{MRM Ratio} = \text{Peak area MRM}_1 / \text{Peak area MRM}_2 \quad [1]$$

The average MRM ratio of the seven calibrators was found to be 1.5 ± 1.9 (\pm SD). This confirmed the absence of matrix effect in the plasma samples and that they are in the acceptable range.

Table 2.3, Absolute and relative matrix effect and recovery of CSUOH0901 in rat plasma

Concentration of QC samples[ng/ml]	Matrix effect		Recovery	
	Absolute	Relative	Absolute	Relative
1.25	5.5%	2.6%	104.0%	99.9%
10	8.1%	5.1%	105.0%	96.1%
80	9.9%	12.4%	104.0%	97.7%

2.3.6. Stability

CSUOH0901 was found to be stable for at least 8 hours at room temperature (bench top) and for 10 hours when post extracted at room temperature and the results were summarized in Table 2.4, The recovery of CSUOH0901 was found to be 112.0% at LQC and 104.5% at HQC levels after 3 freeze-thaw cycles. Stability studies of stock solutions and working solutions of CSUOH0901 and internal standard (JCC76) were performed, respectively by storing them at -20 °C for at least 6 months. The analyte and the internal standard were found to be stable in stock solutions and the results are summarized in Table 2.5.

Table 2.4, Stabilities of CSUOH0901 under various conditions

Stability	Concentration [ng/ml]	Recovery %
Bench top[8hr]	1.25	112.0%
At room temp	80.00	99.1%
Freeze thaw [3 cycles]	1.25	112.0%
	80.00	104.5%
Post extraction	1.25	106.4%
[10hrs] at room temp	80.00	97.8%

Table 2.5, Stabilities of CSUOH0901 and JCC76 Stock solutions after storage at -20 °C for 6 months

Type of Solutions	Concentration	Recovery [%]
CSUOH0901 Stock solution	1 mg/ml	111.8%
JCC76 Stock solution	1 mg/ml	106.0%
CSUOH0901 Working Solution	25 ng/ml	99.6%
	1600 ng/ml	105.0%
JCC76 Working Solution	150 ng/ml	124.6%

2.4. Conclusion

In conclusion, a highly sensitive LC-MS/MS method for the quantitation of CSUOH0901 in rat plasma was developed and validated for the first time. The method developed has a short run time of 18 minutes employing a simple one step sample preparation. The accuracy and precision are lower than 10% and the LLOQ is as low as 0.5 ng/ml. The results from the validation studies illustrated that this method can be used to determine the pharmacological and toxicological profiles of CSUOH0901 in rats in the near future.

2.5. Acknowledgements

We thank Cleveland State University for providing the financial support of this research. We also thank “The National Science Foundation Major Research Instrumentation Grant (CHE-0923398)” which supported the usage of AB Sciex QTrap 5500 mass spectrometer instrument in the current project.

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CHAPTER III

A SIMPLE AND RAPID LC-MS/MS METHOD FOR THE DETERMINATION OF BMCL26, A NOVEL ANTI-PARASITIC AGENT, IN RAT PLASMA

3.1. Introduction

Human African trypanosomiasis, also known as sleeping sickness, is a vector-borne parasitic disease and also a serious health threat to a large number of people living in sub-Saharan Africa where health systems are least effective [1-3]. *Trypanosoma brucei gambiense* (*T. b. gambiense*) and *Trypanosoma brucei rhodesiense* (*T. b. rhodesiense*) are the etiological parasites of sleeping sickness in humans. These parasites live and grow extracellularly in the blood and tissue fluids of humans or cattle, and are transmitted among

hosts by tsetse flies (*Glossina spp*). The disease is always caused by an infected tsetse fly bite and moves through an initial stage, where trypanosomes proliferate in the bloodstream and lymphatic system. The disease can then progress; the trypanosomes will cross the blood-brain barrier and invade the central nervous system eventually. During the second stage, patients will present a variety of neurological symptoms and often exhibit an alteration of the circadian sleep/wake pattern. That is also how the disease is named “sleeping sickness”. Without effective treatment, the disease will lead to coma and ultimately death. If the patients do not receive treatment before the onset of the second phase, neurological damage caused by the parasites is irreversible even after treatment [3, 4].

The current chemotherapy of the human trypanosomiasis relies on only four drugs including Suramin, Pentamidine, Melarsoprol and Eflornithine [5]. The main drawbacks of these drugs are: 1) high toxicity to the hosts, which is mainly due to their poor selectivity to the parasite cells than the mammalian cells; 2) these agents have to be administered via intramuscular or intravenous injections; 3) they have very narrow anti-trypanosomiasis spectrum; and 4) treatment using these drugs needs the high cost of hospitalization. Overall, these drugs are not successful in the treatment of the disease, and there is a general lack of effective, inexpensive chemotherapeutic agents for the treatment of human African trypanosomiasis. Clearly, improved chemotherapeutics with better selectivity to the trypanosomes are needed to effectively treat this disease [4-6].

Tubulin-containing structures are important for many important cellular functions, including chromosome segregation during cell division, intracellular transport, development and maintenance of cell shape, cell motility, and distribution of molecules on

cell membranes [7]. Tubulin is a very attractive target in anti-cancer drug discovery field, and several successful tubulin binders are the first line chemotherapeutic agents in the clinic [8]. Tubulin also plays an essential role during trypanosome cell division. The fast population doubling rate of trypanosomes makes them highly dependent on tubulin polymerization/depolymerization [9]. More importantly, tubulin is very critical for the trypanosome locomotion, which is an essential function for trypanosomes to survive. Tubulin inhibitors not only block the *T. brucei* cell division but will also affect the locomotion function of the flagellum and lead to cell death [10]. These factors indicate that there are potential advantages of tubulin inhibitors for the treatment of trypanosomiasis.

Tubulin is a highly conserved protein. Examination of tubulin sequences from mammalian cells and yeast cells reveals 70% to 90% identity. However, differences in susceptibility to antimitotic agents are known to exist between tubulins from different organisms, suggesting that differences in tubulin structures exist among different species [11]. Based on the differences of tubulin in *T. brucei* and mammalian cells, it is highly expected that selective tubulin inhibitors could be developed. Some microtubule-disrupting herbicides such as phosphoric thioamide herbicide amiprofos-methyl (APM) and dinitroaniline herbicides exhibit activity against protozoan parasites by aiming tubulin as the molecule target [11-15]. Research has been done to optimize these compounds to generate more potent and selective tubulin inhibitors for *T. brucei* [11]. Webovertz's group successfully developed several drug candidates showing promising in vitro anti-parasite activity and selectivity. However, these compounds did not show good in vivo potency due to the poor stability [16]. Nevertheless, these investigations demonstrated the feasibility to generate selective tubulin inhibitors as anti-trypanosomal agents.

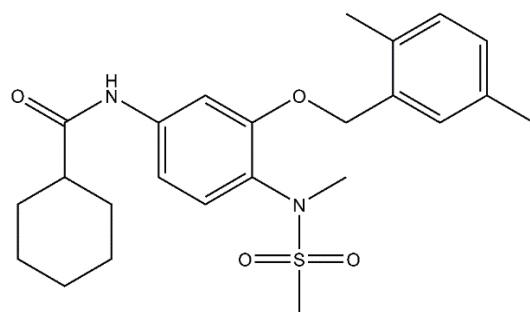
We have developed a class of tubulin inhibitors as anti-cancer agents [17, 18]. These compounds share the same core scaffold, and bind to a colchicine-binding domain on tubulin [17]. Since there is the difference between mammalian and *T. brucei* tubulin, selective tubulin inhibitors for *T. brucei* might be identified within our tubulin inhibitor library. We performed *T. brucei* cell growth inhibition assay with our compounds, and some compounds exhibited a very specific inhibitory effect on *T. brucei* growth, with selectivity index (IC_{50} inhibiting human cancer cell growth/ IC_{50} inhibiting *T. brucei* cell growth) being 5 or more. Compound BMCL26 is identified to be a potential drug candidate. It showed activity against *T. brucei* cell proliferation with an IC_{50} of 1.62 μ M but inhibited mammalian cell growth with an IC_{50} of 55.35 μ M [19]. The selective index is about 34. In addition, the IC_{50} to inhibit *T. brucei* proliferation is at low micromole level that is reachable in blood. The drug may have potential in vivo activity. It is necessary to develop and validate a method to determine the blood drug concentration of BMCL26 for the future pharmacokinetic investigation for this compound. Hence, we focus on the LC-MS/MS method development for compound BMCL26 in the current study.

3.2. Experimental

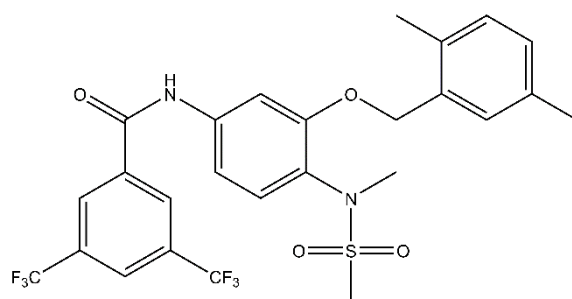
3.2.1. Chemicals and reagents

BMCL26 and JCC76 (Internal standard) were synthesized and purified according to previously published procedures [19, 20] (Fig. 3.1). Methanol (HPLC grade) and acetonitrile were from Pharmco-Apex (Philadelphia, Pennsylvania, USA). Formic acid and ammonium acetate (analytical grade) were purchased from Sigma-Aldrich Chemical

Company (Allentown, Pennsylvania, USA). Deionized water was obtained using a Barnstead Nano pure water purification system with a Nanopure Diamond Pack Organic free DI cartridge from Thermo Scientific (Waltham, Massachusetts, USA). Six individual lots of rat plasma (Sprague-Dawley rat plasmas K2) were obtained from Innovative Research (Novi, Michigan, USA).



[A]



[B]

Figure 3.1, The chemical structures of JCC76 (A), analyte BMCL26 (B).

3.2.2. Calibration standards and quality-control samples

3.2.2.1. Preparation of stock and working solutions

A set of BMCL26 working solutions containing 10, 20, 50, 150, 400, 1000 and 2000 ng/mL were prepared by serial dilution using methanol and 1 mg/mL stock solution. The 150-ng/mL working solution of JCC76 (IS) was diluted from a stock solution of 1 mg/mL in methanol. Stock solutions and working solutions were stored at -20°C and 4°C .

3.2.2.2. Calibration and preparation of quality-control (QC) plasma samples

Calibration plasma samples were prepared by spiking 10 μl of corresponding BMCL26 working solutions in 200 μl of rat plasma (mixture of 6 lots) with drug concentrations of 0.5, 1.0, 2.5, 5, 12.5, 25, 50, and 100 ng/mL. QC samples at three concentrations, 1.25 (low), 10 (mid) and 80 (high) ng/mL, were prepared by adding 10 μL of the appropriate BMCL26 working solution and 200 μL of drug-free plasma. Calibration and QC samples were frozen at -20°C overnight and then treated using the following sample preparation procedure before LC-MS/MS analysis.

3.2.3. Sample extraction

QC samples and blanks were removed from the -20°C freezer and thawed to room temperature. Ten μl of JCC76 working solution were spiked into each 200- μL aliquot of plasma calibrators/QCs/blanks, excepting the double blank, into which 10 μL of

acetonitrile was added. The solutions were then vortexed immediately for 30 secs, after which each sample was deproteinized by adding 800 μ l of 0.1% formic acid in acetonitrile, sonicating for 15 minutes, and centrifuging at $13,000 \times g$ for 15 minutes. The supernatants were then transferred into auto sampler vials for LC-MS/MS analysis.

3.2.4. LC-MS/MS analysis

LC-MS/MS analysis was performed using a 5500 Q-TRAP triple quadrupole, tandem mass spectrometer (AB Sciex, Toronto, Canada) coupled with an electrospray ionizer (ESI) operated in negative ion mode (Framingham, Massachusetts, USA) and interfaced with high performance liquid chromatography (HPLC, Shimadzu, Columbia, Maryland, USA) system that uses auto-sampling and online vacuum degassing. All data acquisition and processing were conducted using Analyst software, version 1.5.2 (AB Sciex).

Analytical separation of BMCL26 was achieved using a Luna C8 [2] HPLC column (50×2.0 mm, 5 microns) with a C8 security-guard cartridge from Phenomenex (Torrance, California, USA). Mobile phase A contained 50 μ M ammonium acetate in 2% Methanol, and mobile phase B contained 50 μ M ammonium acetate in 90% Methanol. Sample aliquots of 5 μ l were injected onto the column and eluted via the following gradient flow: 0-0.6 min, 70% B, 1.6 min, 90% B, 7.5 min, stop (see Table 3.1). The column was equilibrated for 0.5 min before each run. Negative electrospray ionization (ESI) mode was selected, and the MRM (multiple reaction monitoring) function was used for quantification, with the transitions set at m/z 573.3 \rightarrow 493.2 for BMCL26 and m/z 443.2 \rightarrow 79.1 for JCC76 (IS) (Fig. 3.2), respectively. The following ion-source-dependent parameters were

used: nebulization gas [30], heating gas [30], curtain gas [35], ion spray voltage [-4330ev] and temperature [500°C]. Compound-dependent parameters were manually optimized as follows: Declustering potential, -40; entrance potential, -10; collision energy and cell exit potential for both the analyte and internal standard, -30, -100, -13, and -9.

Table 3.1, HPLC gradient program

Minutes	Event	parameters
0 - 0.6	B%	70 [isocratic]
0.6 – 1.6	B%	70 - 90 [liner]
0 – 3.5	Total flow	0.25 ml/min
3.5 – 3.7	Total flow	0.25 - 0.6 ml/min
1.6 – 5.5	B%	90 [isocratic]
5.5 – 5.6	B%	90 – 70 [liner]
3.5 – 5.9	Total flow	0.6 ml/min
5.9 – 6.0	Total flow	0.25 – 0.6 ml/min
5.6 – 7.5	B%	70 [liner]

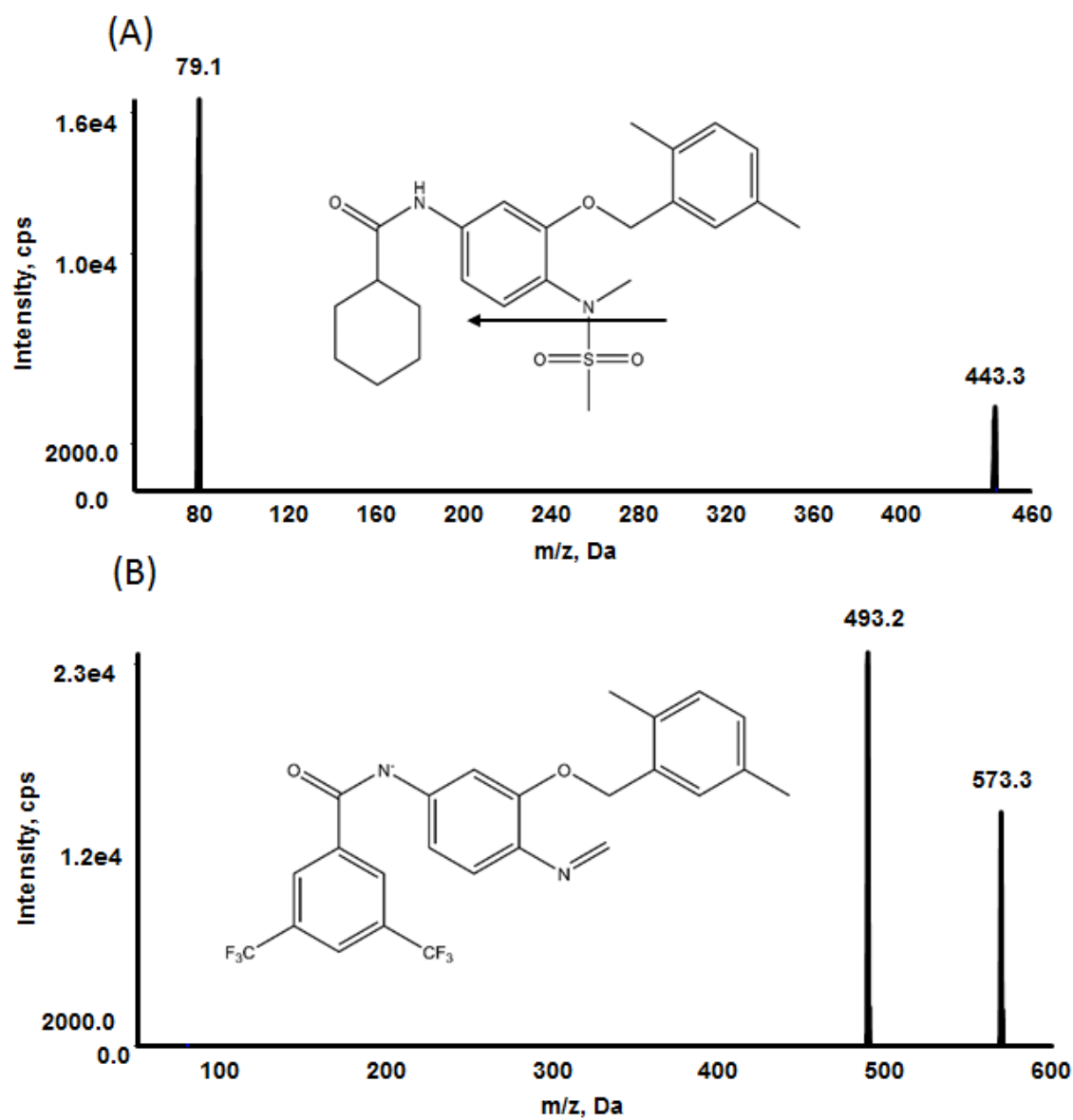


Figure 3.2, Precursor/product ion spectra and proposed fragmentation pathways for internal standard JCC76 (A) and analyte BMCL26 (B).

3.2.5. Analytical method validation

The LC-MS/MS assay was fully validated according to the Food and Drug Administration (FDA) Bioanalytical Method Guidelines [21] and other references [22-24]. The entire assay was validated for linearity, accuracy, precision, selectivity, extraction recovery, matrix effect, and stability.

3.2.5.1. Linearity and calibration

Eight concentrations of BMCL26 (0.5, 1.0, 2.5, 5, 12.5, 25, 50, and 100 ng/mL) were selected for the plasma calibration curve (n=2 for each of the eight calibrators, average for each calibrator plotted). A weighed linear regression, using 1/x as the weighing factor, was used to calculate the slope and correlation coefficient of the calibration curve.

3.2.5.2. Accuracy and precision

To determine the Intra- and inter-day accuracy and precision of the assay, we tested five replicates of BMCL26 at the LQC, MQC, and HQC using 1.25, 10 and 80 ng/mL. Intra-assay and inter-assay precisions were defined as relative standard deviations (RSD) between replicate measurements. Accuracies were calculated using the following formula:
$$\text{accuracy (\%)} = (\text{experimental concentration} - \text{spiked concentration}) / (\text{spiked concentration}) \times 100$$
. The criteria for data acceptability included accuracy and precisions within $\pm 15\%$ of the nominal value and the RSD.

3.2.5.3. Extraction recovery and matrix effect

The extraction recoveries and matrix effects of BMCL26 for three concentrations of QC samples (low, 1.25 ng/mL; medium, 10 ng/mL; and high, 80 ng/mL) were determined by comparing peak areas of analyte-spiked plasma aliquots before extraction to peak areas of analyte-spiked solutions extracted from blank plasma and comparing the response of solutions spiked with analyte after extraction to the response of analyte dissolved in the mobile phase.

3.2.5.4. Stability studies

The stabilities of BMCL26 in rat plasma samples were determined using two distinct QC standards (1.25 and 80 ng/mL), which were tested after 8 hours at room temperature and after two months at -20°C ; freeze-thaw stabilities were determined using three freeze-thaw cycles over a three-day period. Short-term stabilities were also determined for post-preparation QC standards stored at room temperature for 10 hours. The QC results obtained after storage were compared with spiked concentration values by determining the percentage ratios of experimental values divided by spiked values.

The stabilities of stock and working solutions for both analyte and internal standard were also evaluated. Stock solutions of analyte and IS were stored at -20°C for 9 months. From both stored and fresh stock solutions, two QC standards (1.25 and 80 ng/mL) were prepared for the analyte, to which 7.5 ng/mL IS (JCC76) was added, and the experimentally determined concentrations of BMCL26 and JCC76 were compared (n=3 for each).

3.3. Results and discussion

3.3.1. Optimization of mass spectrometric conditions for quantitation

The negative ionization mode was selected for both BMCL26 and JCC76 (IS) detection in this study because the analyte produced a much stronger signal for negative ionization compared to positive ionization. It was found that analyte and IS solutions prepared in methanol-water (9:1, v/v) yielded stronger signals compared to solutions prepared in acetonitrile-water (9:1, v/v). Adding ammonium acetate to the methanol-water solution substantially increased BMCL26 signals. Therefore, a gradient flow of methanol-water-ammonium acetate was chosen for the HPLC mobile phase. Figure 3.2 showed the parent ion spectra for both BMCL26 and IS. The highest fragment-ion signals were obtained by fine-tuning the collision energy, spray voltage, and ion source temperature. Based on our ionization and fragmentation optimization results, we chose MRM transitions of m/z 573.3 \rightarrow 493.2 for BMCL26 and 443.2 \rightarrow 79.1 for IS for quantification, as these product ions yielded strong and stable signals.

3.3.2. Optimization of HPLC conditions

The gradient flow of the mobile phase, which was used with different flow rates, was as follows: A: 50 μ M ammonium acetate in 2% methanol and mobile phase; B: 50 μ M ammonium acetate in 90% methanol (see Table 3.1). Because the C-18 column had a carry-over problem with BMCL26, a C-8 column was used instead. The C-8 column effectively resolved the carryover problem and also yielded a better peak shape. Thus, BMCL26 and

IS were separated using a C-8 column with a flow rate of 0.25 ml/min for 3.5 min, after which the flow rate was increased to 0.6 ml/min for column clean up and re-equilibration. Retention times were observed at 2.48 min for BMCL26 and 3.12 min for IS (Fig. 3.3B). The total run time was 8 min.

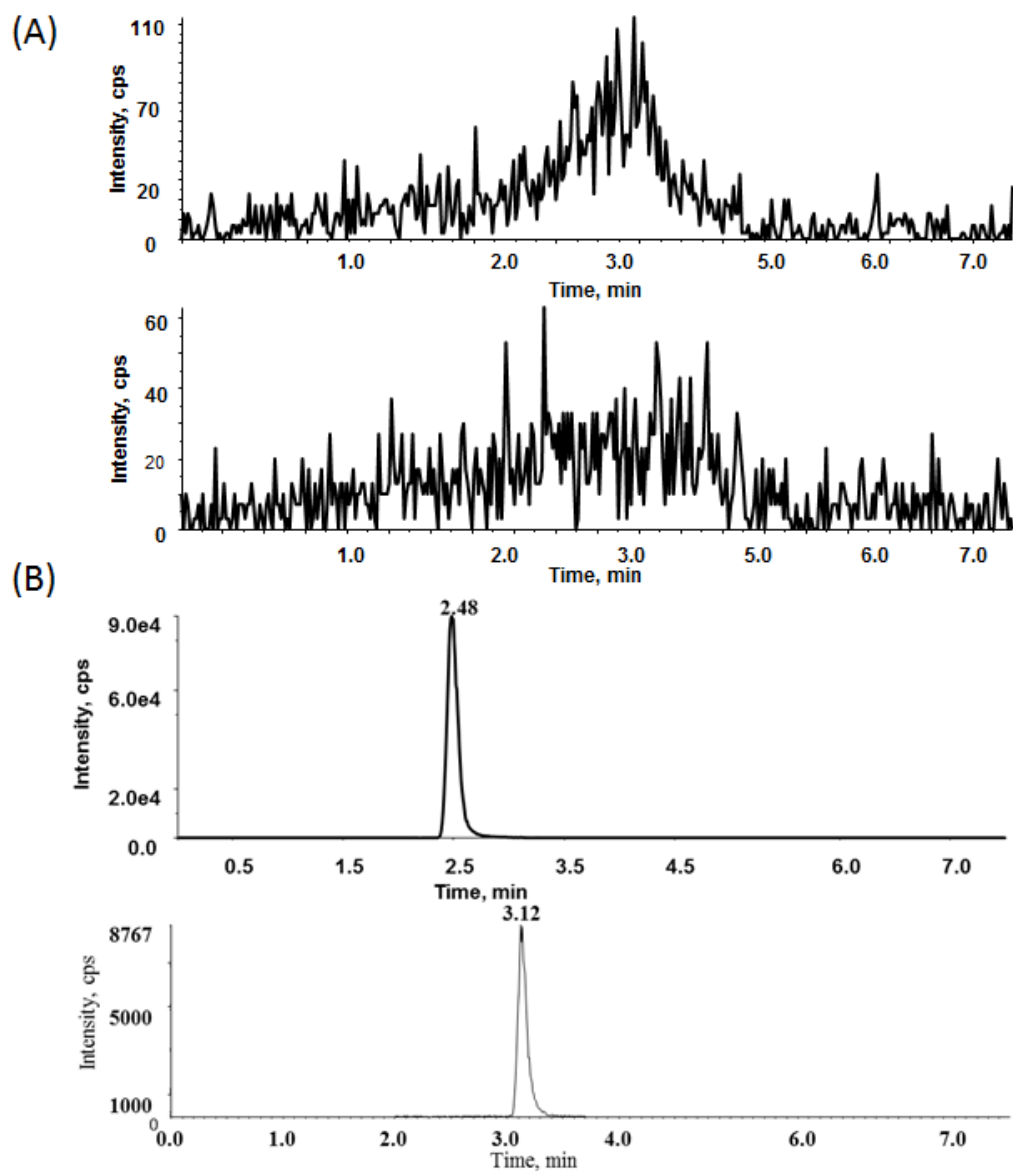


Figure 3.3, (A) MRM chromatograms of blank rat plasma in both IS and analyte windows (B) IS JCC76 (10 ng/ml, 3.12 min) and BMCL26 at LLOQ level (0.5 ng/ml 2.48 min).

3.3.3. Linearity, sensitivity, selectivity, and LLOQ

Using the concentrations of BMCL26 in the working solutions, plasma calibration curves were constructed over a concentration range of 0.5 – 100 ng/mL. Linearity results showed a quadratic fit for BMCL26 using an eight-point calibration curve (0.5, 1, 2.5, 5, 12.5, 25, 50, and 100 ng/mL) with JCC76 (7.5 ng/mL) as the internal standard in the plasma samples. Excellent linearity was obtained with a correlation coefficient (r^2) of 0.9993. The linear regression equation was $y = 0.073x - 0.0085$. This method exhibited high selectivity and displayed no interfering peaks in six different blank plasma samples from different sources. Using the calibration curve, the LLOQ of the method was determined to be 0.5 ng/mL. The accuracy and precision were determined for each lot of plasma at the LLOQ. The data were summarized in Table 3.2.

Table 3.2, Accuracy and precision of BMCL26 calibration standards in rat plasma (n=5, pooled plasma samples)

Nominal Concentration	Determined Concentration	Accuracy	Precision
[ng/mL]	[ng/mL]	[%RE]	[%CV]
0.5	0.47±0.03	-7.46	5.59
1	0.99±0.02	-0.22	2.09
2.5	2.54±0.05	1.40	2.01
5	5.14±0.27	2.80	5.24
12.5	12.75±0.31	2.00	2.41
25	25.27±0.73	1.08	2.90
50	51.03±0.97	2.06	1.90
100	98.26±0.99	-1.74	1.01

3.3.4. Accuracy and precision

Intra- and inter-assay accuracies (%RE) and precisions (%CV) were evaluated by analyzing five replicates of low, medium, and high QC standards. As summarized in Table 3.3, the assay's Intra- and inter-day relative errors were 0.62 and 11.36%, respectively, and the assay's Intra- and inter-day precisions were 0.84-3.47%, respectively. These values were within acceptable limits according to FDA guidelines.

Table 3.3, Inter and intra-assay accuracy and precision of BMCL26 in rat plasma

Intra- assay					Inter- assay			
Normal	Determined	Accuracy	SD	Precision	Determined	Accuracy	SD	Precision
[ng/ml]	[ng/ml]	[%RE]		[%CV]	[ng/ml]	[%RE]		[%CV]
1.25	1.20	11.36	0.02	1.72	1.36	9.12	0.04	3.38
10	10.20	2.00	0.16	1.64	10.20	2.00	0.35	3.43
80	80.70	0.87	0.68	0.84	80.50	0.62	2.80	3.47

3.3.5. Extraction recovery and matrix effect

It was found that protein precipitation was a good way to extract BMCL26 from plasma. Initially, we used 80% acetonitrile to precipitate plasma proteins, but a strong matrix effect was observed. Consequently, we utilized pure acetonitrile to precipitate proteins with 0.1% formic acid (for deproteinization). The latter precipitation conditions eliminated the matrix effect and achieved symmetrical chromatographic peak shapes. The absolute and relative extraction recoveries were in the range of 90.16-105.00%. The results were summarized in Table 3.5. As shown in Table 3.4, the absolute and relative matrix effects of BMCL26 from six different rat plasma samples at three QC concentrations low (1.25 ng/mL), medium (10 ng/mL), and high (80 ng/mL) ranged from 101.30 – 110.10%.

Table 3.4, Absolute and relative matrix effect of BMCL26 in rat plasma

Concentration of QC samples[ng/ml]	Absolute		Relative	
	Mean matrix effect	%CV	Mean matrix effect	%CV
1.25	105.00 ± 1.07	1.02	108.00 ± 5.15	4.77
10	107.00 ± 6.06	5.66	110.10 ± 8.99	8.17
80	105.00 ± 3.86	3.68	101.30 ± 4.78	4.72

Table 3.5, Absolute and relative extraction recovery of BMCL26 in rat plasma

Concentration of QC samples[ng/ml]	Absolute		Relative	
	Mean extraction recovery	%CV	Mean extraction recovery	%CV
1.25	104.00 ± 4.89	4.70	98.10 ± 9.73	9.92
10	105.00 ± 4.99	4.75	95.96 ± 3.61	3.76
80	96.30 ± 3.00	3.12	90.16 ± 2.87	3.18

3.3.6. Stability

The stability of BMCL26 was determined by comparing the mean peak area ratios of BMCL26 to IS in low (1.25 ng/mL) and high (80 ng/mL) QC samples to those of freshly prepared QC solutions (containing the same concentrations), expressed in terms of recovery. As shown in Table 3.6, the receives of LQC and HQC samples were 103.46 – 103.20%, 98.13 – 105.20%, and 101.86 – 107.54% for bench top conditions, after 3 freeze-thaw cycles and post extraction at room temperature for 10 hours, respectively. The stabilities of working solutions of BMCL26 and internal standard (JCC76), stored at 4 °C for at least 6 months, were determined to be 99.60 – 105.00% and 115.60% for the two QC standards tested (1.25 and 80 ng/mL), to which 7.5 ng/mL IS was added (using the stored stock solution). These stability results showed no significant deviations in BMCL26 quantification under the experimental conditions used.

Table 3.6, Stability of BMCL26 in plasma samples

Storage conditions	Concentration	Recovery
	[ng/ml]	%
Bench top[8hr]	1.25	103.46
At room temp	80.00	103.20
Freeze-thaw [3 cycles]	1.25	98.13
	80.00	105.20
Post extraction	1.25	101.86
[10hrs] at room temp	80.00	107.54

3.4. Conclusion

This innovative method offers several advantages for assaying BMCL26 in rat plasma including simplicity, cost effectiveness, accuracy, and precision were below 11% and 3%, with high sensitivity and selectivity. To our knowledge, this is the first time this method was used. This assay employed a simple protein precipitation procedure for plasma sample preparation. The LLOQ was as low as 0.5 ng/mL. The results from the validation study illustrated that this method can be used to determine the pharmacological and toxicological profiles of BMCL26 in rats in the future.

3.5. Acknowledgements

The authors acknowledge the following sources of support for this work: NIH grant R15AI 103889 (B. Su) and the National Science Foundation Major Research Instrumentation Grant (CHE-0923398).

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Part - II

CHAPTER IV

REVIEW OF HCC AND DNA METHYLATION

4.1. Introduction

DNA Methylation is the covalent chemical modification resulting in addition of methyl group at 5th carbon position of the cytosine ring. DNA methylation is a naturally occurring event in both prokaryotes and eukaryotes. In prokaryotes, they majorly play a role of protection of the host DNA from restriction endonucleases produced to destroy foreign DNA [1]. However, they have a complicated role in eukaryotes like gene imprinting, X chromosome Inactivation, embryonic development and cell cycle

regulation. When we consider the classic view of DNA methylation, it varies according to the developmental stages namely pre and post developmental processes. The former pattern associates the methylation processes into active or passive methylation whereas the latter involves its maintenance based on DNMT's [2]. DNA methylation errors are potentially reversible and as a result aid in Pharmacological changes.

4.1.1. DNA methylation and carcinogenesis

Aberrant changes in the DNA methylation promote disease state. Aberrant DNA methylation is found in two distinct forms namely hyper methylation and DNA hypomethylation. Hyper methylation is usually associated with the gene inactivation involving CpG islands and hypo methylation involving repeated DNA sequences of nuclear elements [3]. These two processes are independent processes [4].

4.1.2. Hypermethylation in cancer

It is one of the most studied epigenetic changes in cancer. DNA hyper methylation typically occurs in the CpG islands. The promoter site and transcription site are included within the CpG islands and when hyper methylation occurs at these islands, the expression of gene is totally repressed leading to gene inactivation [5].

4.1.2.1. CpG islands

DNA is made up of four bases namely Adenine, Guanine, Cytosine and Thymine. Among these four bases, the numbers of dinucleotide combinations possible are 16 [6]. Out of which, the cytosine methylation occurs in 5'CG 3' dinucleotide combination. Based on the percentage of these combinations, the frequency of these dinucleotides should be 10% but only occur around 5% [7]. Methylation isn't uniform through the human genome, containing both methylated and unmethylated segments. Unlike the rest of the genome, CpG islands are interspaced on an average of 100kb and ranging up to 5kb are smaller regions of unmethylated DNA which are GC rich, Guanine Adenine content above 0.5 and having distinctive properties without any suppression of the occurrence of dinucleotide CpG. It is known that human genome contains around 29000 CpG islands and is associated with about 60% of mammalian genes, mostly in promoter and first exon region of genes. In normal, healthy tissues CpG islands are unmethylated but these are methylated to various extents in cancer [8]. There are different protective mechanisms that prevent the hyper methylation at these CpG islands like demethylation, changes in chromatin structure, active transcription and timing of replication [6]. As the figure 1.1 indicates, DNA methylation is a reversible reaction and the active chromatin recruit's DNA demethylases, the enzymes responsible for DNA demethylation and inactive chromatin recruits DNMT's. Therefore, if the chromatin structure is maintained DNA methylation is maintained. Hyper methylation is region specific and the local changes around this specific region of genes inhibit them from interacting with the demethylases, protecting them from demethylation.

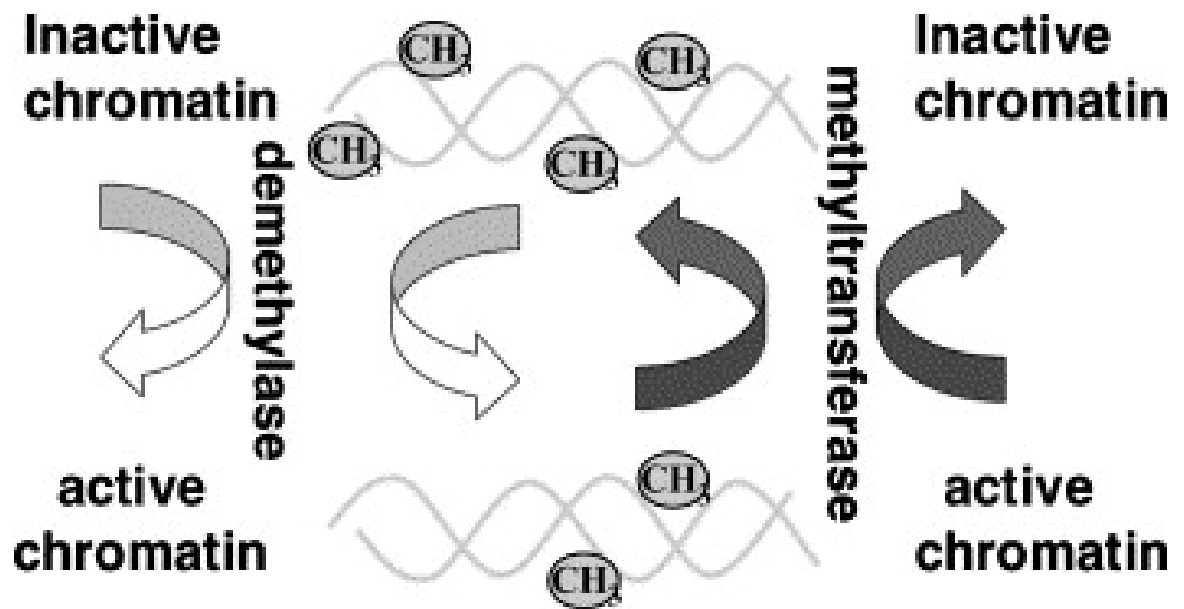


Figure 4.1, The methylation state of a gene is equilibrium of methylation and demethylation reaction [9].

4.1.2.2. Global hypo methylation in cancer

Global DNA hypo methylation was the first identified epigenetic event in cancer. Hypo methylation is the loss of methylation and this loss is quite predominantly seen as they are quantitative in nature and affect a variety of repetitive sequences which are dispersed throughout the genome. Usually, the pericentric regions of chromosome 1-16 are hypo methylated, with most cases occurring in regions with sparsely distributed CG sequences. Hypo methylation plays a role in cancer by various mechanisms like instability of the chromosome [10], activation of the long interspread nuclear elements called retrotransposons in the genome [11]. Hypo methylation of these transposons, which are usually latent, may lead to oncogenesis by transcriptional activation and sometimes may express themselves on the adjacent genes [12].

4.1.3. DNA methylation machinery

Based on the methylation patterns of CpG dinucleotides on both strands, DNA methylation can be either Denovo Methylation or Maintenance Methylation. Denovo Methylation occurs, when the CpG dinucleotides on both the strands are unmethylated and has an important role in cell growth, differentiation and tumor genesis. Maintenance methylation occurs when CpG dinucleotides on only one strand are methylated [9]. It mainly copies the DNA methylation patterns but does not introduce any different methylation patterns. As the figure 1.2 suggests, DNA methylation machinery can be classified into two ways namely, transcriptional repressor machinery to the corresponding promoter CpG island which contribute to tumorigenesis by silencing of

TSG's and the Transcriptional Activation machinery for the active expression of the oncogene.

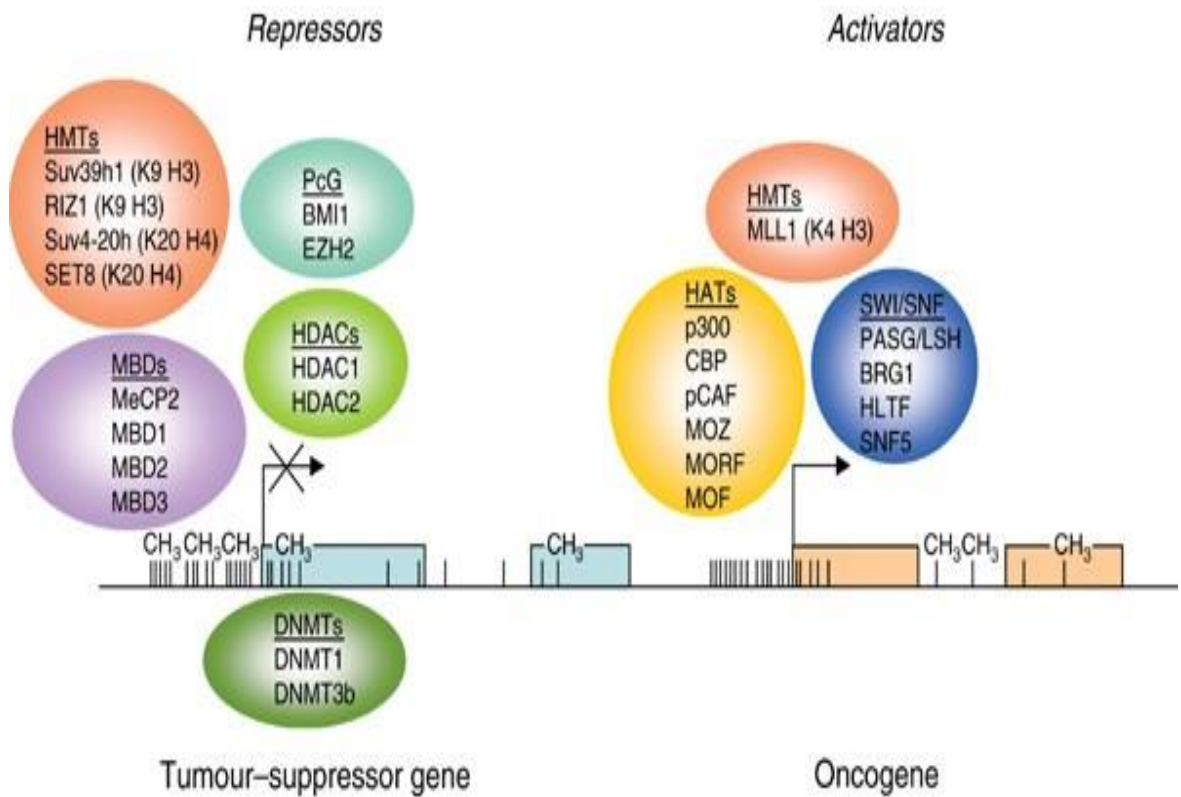


Figure 4.2, Epigenetic provides a new generation of oncogene and tumor-suppressor genes

Transcriptional Repressor Machinery include DNA methyl transferases, Methyl CpG Binding proteins, histone methyltransferases for lysine 9 of histone H3 (HMT K9 H3), histone deacetylases and polycomb. Transcriptional Activator machinery includes HAT's, HMT's and others. Of these, epigenetic machinery DNMT's, MBD's and HAT's play an important role in DNA methylation.

4.1.4. DNA methyltransferases [DNMTs]

DNA methyl transferases are the enzymes that catalyze the transfer of the methyl group from the methyl donor S-adenosyl methionine onto the DNA and release S-adenosyl homocysteine [13]. DNMTs are the critical proteins involved in establishing proper control of epigenetic information. In mammals, four different types of DNMTs are seen DNMT1, DNMT2, DNMT3a and DNMT3b. The balance of DNMT's is very important to prevent cell transformation. The DNMT's in conjugation with accessory proteins like DNMT3L are mainly responsible for DNA methylation at various stages like embryogenesis and development of somatic tissue [14].

DNMT 1 is the most abundantly found among DNA methyl transferases. It is useful in maintaining the post replicative DNA methylation patterns. It is useful for maintaining the methylation pattern during cell division. The DNA Methylation patterns are passed on from the parental strand to daughter cells and DNMT1 maintains this maintenance methylation [15]. It also plays an important part in Denovo methylation [16]. They maintain gene silencing in cancer cells, by cooperative interactions between DNMTs especially DNMT1 and DNMT3b. DNMT 2, is not involved much in DNA methylation.

However, it can contribute to cancer through different pathways related to RNA. DNMT3b is mainly involved in Denovo methylation. It is also a part of several complexes. It inhibits gene expression by forming a transcription repressive complex. DNMT3B leads to demethylation when it is deleted and with DNMT1 and maintain the global methylation [17].

4.1.5. Methyl CpG binding domain proteins

MBD proteins are considered interpreters of DNA methylation. They are important between DNA methylation and genes involved in histone modifications. All of them bind to the CpG sites with the exception of MBD3 [18]. Twelve different types of Methyl CpG proteins have been identified of which MBD1, MBD2, MBD3, MBD4 and MeCP2 are the most important members [17]. They play a role in the pathophysiology by a number of mechanisms. They associate with the CpG island promoters of the Tumor suppressor genes and lead to transcriptional silencing. MBD4 is different when compared to other MBDs as it is a thymine glycolase and it helps in DNA repair protein, as the Glycolase domain removes thymidine from T: G mismatches [19].

4.1.6. Histone modification enzymes

Histones are used to store the epigenetic information through modifications of the amino terminal protruding tails. These modifications generally include acetylation, phosphorylation, and methylation. Histone Modification Enzymes involved are histone

acetyl transferases (HATs), Histone methyltransferases (HMTs) and histone deacetylases (HDACs). HATs are involved in the Acetylation of histone lysine's and HMTs aid in the deacetylation.

4.2. Hepatocellular carcinoma

Primary Liver cancer can be distinguished into three main types namely Hepatocellular Carcinoma which occurs from Hepatocytes, Duct cell Carcinoma and cholangiohepatoma which occurs from intrahepatic bile ducts. Clinically there is not much difference between them since they spread throughout the liver [20]. Primary Liver Cancer is unique when compared to all the malignancies because of its worldwide distribution, association with cirrhosis, spontaneity of the tumor and inherent difficulties [21]. Primary Liver Cancer is the fifth most common cancer in the world and second rated cancer with most deaths [22]. Hepatocytes, the parenchymatous cells which are chiefly responsible for its functioning and their uncontrolled division leads to primary liver cancer in most cases. Hepatocellular Carcinoma is the predominant type of liver cancer and also called as hepatoma based on its occurrence and it accounts for about 85-90% primary liver cancers [23]. HCC is the fifth most common cancer in men and eighth most common in women. Hepatocellular Carcinoma is usually asymptomatic, with Cirrhosis being the major condition present in about 80-90% of them and by the time of diagnosis it is usually advanced leaving with a 5-year survival rate. Hepatocellular Carcinoma have different growth patterns with some starting as single tumor and grows large before spreading and others appear as small spots in various areas. It is very important to diagnosis HCC early to improve the survival rate.

4.2.1. Epidemiology of hepatocellular carcinoma

Several epidemiological factors like age, sex, ethnicity, distribution of risk factors influence HCC. Geographically speaking, greater number of the cases of HCC is found either in sub-Saharan or Asian populations. In United States, the rates of incidence increased at a two-fold rate between 1985 and 2002. When you compare ethnicity among different regions, Asians are twice susceptible to HCC than Americans. This might depend on the differences in attainment of the risk factors of HCC. The age is an interdependent epidemiological factor based on various factors like sex, age of infection and so on. Hepatitis B Virus usually infects at younger age when compared to HCV. Throughout the world, age of incidence of HCC in men is approximately 5 years lower when compared to that of women. Sex of an individual highly influences the incidence rate because, throughout the ratio of incidence between male and female is either two fold or four fold with male dominating. This again might be due to various factors like exposure to viral factors, alcoholism and smoking with largest difference in ratios seen in European populations. Studies conducted state that men have higher BMI and higher level of androgenic hormones might be a reason for higher incidence of HCC. HBV and HCV are the two most dominant risk factors in HCC. However, their distribution varies from place to place. Globally, HBV risk factor is more dominant. In places like Japan HCV show greater dominance than HBV.

4.2.2. Risk Factor for hepatocellular carcinoma

The risk factors of HCC can broadly be categorized into pathological factors, physiological factors, exposure to environmental toxins and dietary factors [23]. The physiological factors like geography, age and sex are mostly patterns of the pathological factors mostly HCV, HBV and alcohol liver disease [24]. The physiological factors for HCC incidence increase with age and males have greater chance of Incidence than females. The pathological factors can be categorized into Hepatitis B virus and Hepatitis C virus. Liver Cirrhosis can be considered to be the main precursor because about 70% have HBV related Cirrhosis when compared to the 20% HCV related Cirrhosis [25]. Various studies show that incidence of HCC in patients with chronic Hepatitis or Cirrhosis is greater when compared to the patients with normal transaminases [26]. HCV does not play a direct role in HCC. However, it leads to development of Cancer through Cirrhosis. Exposure to environmental Toxins like Aflatoxin, which is a product of *Aspergillus* fungus, on the legume crops is less developing countries and on dietary intake of them leads to development of HCC [27, 28]. Alcohol does not have any particular mutagenic properties. Effect of Consumption of Alcohol is usually dose dependent and leading to HCC through Cirrhosis. It was proved in a Cirrhosis study that chances of HCC are 13 times greater in people consuming alcohol when compared to people who stay away from it [29]. Smoking is also an important factor in HCC, with studies showing a contribution of about 50% in HCC [30]. Few of the rarer factors which influence HCC are hemochromatosis which is a genetic factor and few metabolic diseases like Diabetes type-II, glycogen storage disease type 1 and alpha-1 antitrypsin deficiency [31].

4.2.3. Molecular mechanisms of hepato- carcinogenesis

The pathophysiology of HCC is mainly based upon cirrhosis. Different molecular mechanisms inhibit the regenerative capacity of the liver at cirrhotic stage and these mechanisms tend to increase the rate of carcinogenesis at cirrhotic stage. They can be classified into cell intrinsic alterations and cell extrinsic alterations [23]. Cell intrinsic alterations involved are the shortening of the length of telomere which restricts the proliferation of hepatocytes [32, 33], thereby leading to induction of chromosomal fusion by activating DNA repair pathways [34, 35]. Cell extrinsic alterations involved mainly are the decrease in the proliferation of the primary liver cells and changes in the micro and macro environment, thereby promoting risk of cancer Figure 4.3.

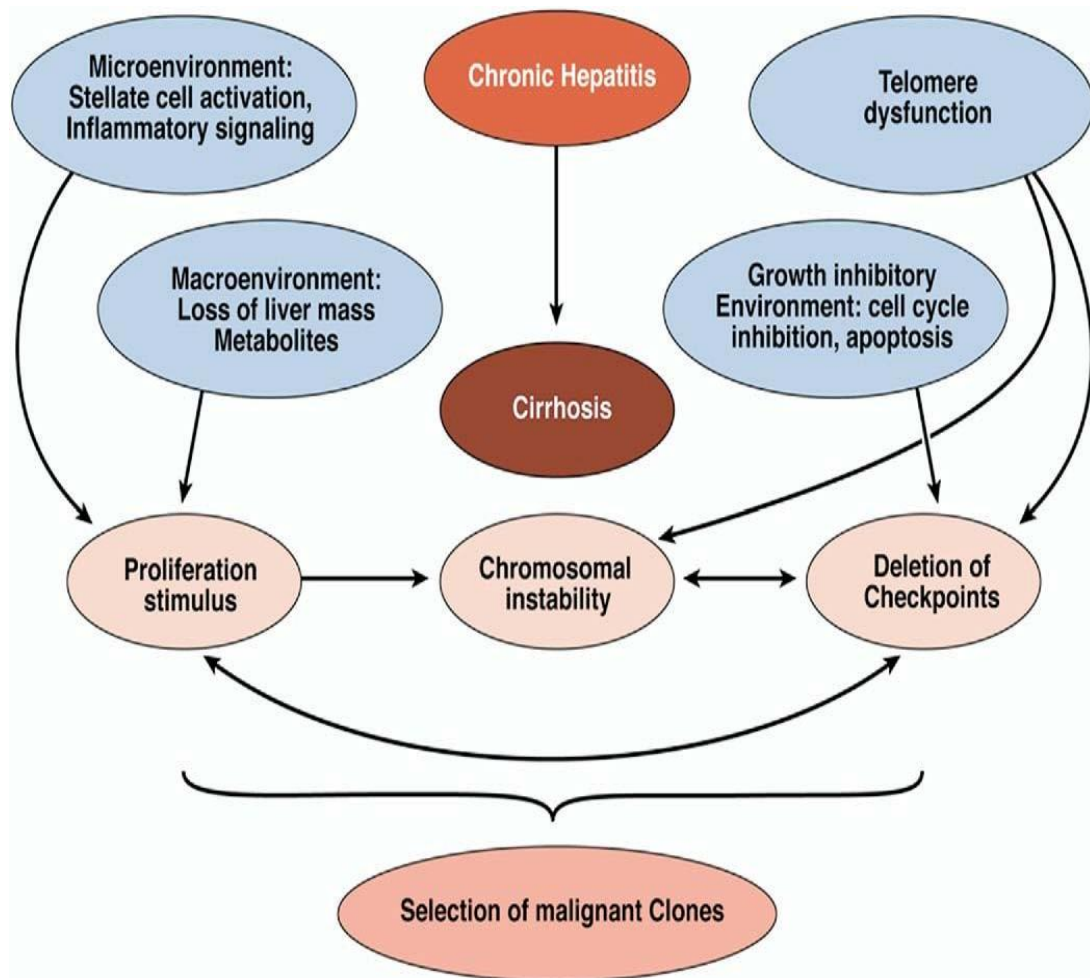


Figure: 4.3, Molecular mechanisms of hepatocellular carcinoma at the cirrhotic stage [23]

4.2.4. Staging and diagnosis of hepatocellular carcinoma

The method developed by Okuda et al is the most commonly used staging system presently. This system evaluates the albumin levels, size of the tumors and concentration of bilirubin of patients. However, it is ineffective at early stages of the disease. Several prognostic staging systems are currently in clinical use like Barcelona Clinic Liver Cancer, Tumor node metastasis which although advanced does not consider underlying Cirrhosis. Although they all differ on the way of evaluation, they are useful in better understanding of diagnosis.

HCC Tumor is categorized by AJCC, into various stages based on the clinical status of the patient, the size of the tumor, the number of lesions. Stage I and II with the tumor showing some Invasiveness in the blood vessels and forming single to multiple tumors. In stage III a, multiple tumors are formed which tend to increase in size and these tumors extend to the hepatic vein by stage III b and reach the visceral peritoneum by Stage III c. In stage IV a and IV b leads to initially regional to distant metastasis [36, 37].

Diagnosis of HCC involves differential Diagnosis of well differentiated HCCs from lesions and poorly differentiated HCCs from malignancies similar to HCC outside liver. Usually well differentiated HCC's from dysplastic nodules and poorly differentiated HCC's from metastatic tumors, chlorangiosarcoma [38]. Diagnosis is mostly possible only when symptoms like weight loss or sometimes much more severe like liver failure are seen and usually suggesting presence of tumored liver [39]. Some of the common methods of diagnosis include imaging like CT scan, MRI. The noninvasive nature, fewer costs make imaging more preferable though they are late in detecting only until large tumors are formed [40]. The confirmation of the pathology of HCC play a role in

diagnosis and biopsy is a method of doing it. However, it also chances of spreading the disease.

4.2.5. Current treatment options

Surgical resection is the preferred method for very early HCC. It is usually indicated in patients without cirrhosis and portal hypertension. Patients with preserved liver function are resected. The only problem it faces is recurrence. However, in combination with adjuvant therapy it can be controlled. Liver transplantation is another method for treatment of early HCC and is suitable for patients without proper functioning of liver and any extra hepatic spread or vascular invasion and nodules of size less than 5cm. However, the demand of livers and waiting time is a problem. If the waiting time is greater than 12 months 25% more chances of tumor being spread. Ablation is a method with similar recurrence chances and survival rates when compared to Surgical Resection. It is effective when nodules are below 2cm [41]. Percutaneous ethanol injection has been the most successful technique of ablation with little adverse effects. It is time taking and ineffective in tumors larger than 3cm. RFA solves most of these issues of the PEI with greater side effects [39]. Some other common ablation techniques are microwave and cryoablation. For patients, with Intermediate HCC and multi nodular tumors without any invasion and extra hepatic spread, Transcatheter Arterial Chemo embolization and Trans-arterial embolization with a radioactive isotope Y90 was used. For patients with Advanced HCC, systemic therapy with Sorafenib, a multi kinase inhibitor is mostly used and few others like Sunitinib, brivonib and being investigated [42, 43].

4.2.6. Current trends in research of biomarkers for hepatocellular carcinoma

HCC is usually asymptomatic and biomarkers therefore act as measurement tools for diagnosis of the disease, its progression and help to target a proper therapeutic treatment. Different types of Biomarkers are presently used worldwide. Alpha–Feto protein is the most commonly used biomarker presently and can be called as a gold standard, it is pretty much useful in patients with known risk factors as its increase is directly in correspondence with the development of HCC [44] [45]. However, it does not correlate much to the prognostic factors of HCC.

Different types of immuno histochemistry markers are useful as diagnostic markers as they use immunochemical staining to distinguishing well differentiated HCC. Some of them are HSP70, Glypican 3. These usually appear and reappear during tumor genesis and usually used in combinations [46]. Some of the IHC markers used in prognosis are Ki67, Survivin and E-Cadherin. Enzymes and Iso-enzymes used as biomarkers like Des-Gamma-Carboxy Prothrombin which when used with AFP increases the sensitivity, gamma glutamyl tranferase also is used in combinations with AFP and DCP increases the sensitivity [47]. Several growth factors like fibroblast growth factor, epidermal growth factor receptor family and hepatocyte Growth Factor are used. Hepatocyte Growth factor plays an active role as biomarker in prognosis and recurrence. It plays a role in HCC through the pancrine system involving the receptor c-met. Circulating Nucleic acids mainly mRNA, like GGT mRNA, IGF-2 mRNA are analyzed in various pathological and physiological conditions. Micro RNAs provide new insights into HCC carcinogenesis and therefore used in prognosis and diagnosis of the disease. Depending upon the etiology of the HCC and the molecular abnormalities make it pretty difficult to

find a biomarker with good specificity and sensitivity. These are the currently used markers based on the different molecular pathways of HCC and these show great amount of significance and on further research can be used to improve the prognostic values and assist in determining the proper mode of therapy.

4.3. DNA methylation analysis techniques

DNA methylation analysis techniques can be categorized based on chemical modification of molecules, the precipitation of methylated DNA by proteins which interact with the methylated cytosines [48]. It is important to choose a protocol on the basis of several factors like sensitivity, cost, specificity and the desired results. In the precipitation techniques, complexes are formed between the proteins and the methylated DNA. These proteins bind and enrich the methylated DNA. Several protocols use different proteins, as in MIRA protocol, a complex of MBDs with glutathione γ -S-transferase isolates the methylated DNA [49]. MBD2 and MeCP2 methyl binding domain of MBD2 are the most commonly used in isolating methylated DNA [50]. MBDs are not specific and are affected by the methylation density of DNA [51]. Lack of Specificity and sensitivity limits the precipitation techniques usage.

DNA methylation Analysis techniques using methylation sensitive restriction enzymes which are sensitive to the cytosine methylation in CpG can be used [52]. This method involves the division of DNA into two samples and then digests one with enzyme sensitive to the DNA and one without sensitive to DNA methylation and later the missing cuts of enzymes and methylation are compared [48]. However, the incomplete digestion,

buffer conditions, over dependence on the sequence of Restriction site and the distribution limits its sensitivity. Microarrays techniques are generally used in combination with few analysis techniques. High resolution arrays like oligonucleotide arrays have been used in analysis [53]. Some of the arrays used are SNP's [54]. They cannot quantify the methylation level accurately and therefore acts as a drawback. Methylation Bead chip technology is the currently used microarray technique and it can effectively overcome the drawbacks [55]. The techniques which focus on the alterations of DNA depend mainly on the conversion of the methylated DNA by the treatment with sodium bisulfite. The figure 4.4, gives a small description of methods of the DNA Methylation Analysis techniques presently used.

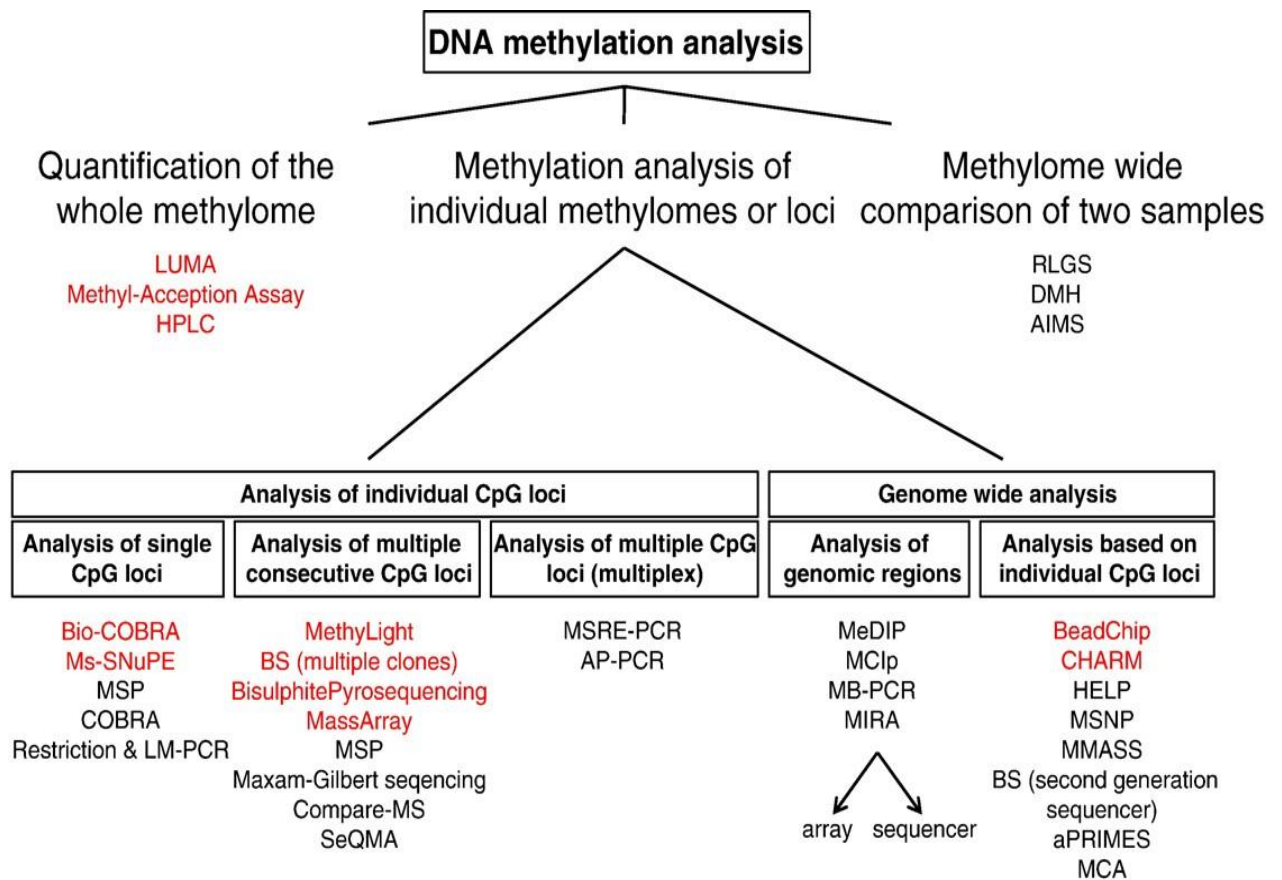


Figure 4.4, DNA methylation analysis techniques

4.3.1. Methylation-specific PCR [MSP]

MSP was one of the early changes which involved Bisulfite Conversion. It was introduced by Herman and colleagues [56]. It is a standard PCR reaction used to analyze the methylation status of CpG islands. In this reaction, distinct methylation primer sets for the sequence of interest are used like, the unmethylated primers specific for the unmethylated DNA whereas the methylated primer specific for methylated DNA. The unmethylated cytosine's are converted to thymine's and methylated cytosine's are unconverted and therefore the primers designed should include CpGs of interest. When compared to other DNA methylation techniques MSP exhibits several advantages based on the simplicity of the procedure, time taken for and lack of need of any specific equipment. It is also less expensive and the presence of larger number of the CpG sites in the CpG islands. These factors aid the use of MSP in larger number of samples with a better sensitivity. It is also used in the prevention of unwanted non tumor cells in the sample, if a uniformly opposite pattern is observed by the tumor cells thereby increasing the detection [57]. However, it is difficult to obtain good capability as it is a very laborious process. It depends on various critical factors like Bisulfite Conversion, designing of the primer and PCR.

4.3.2. Bisulfite conversion

The existence of 5-methyl cytosine in the DNA is a very important area of study in the present genomic era. One of the most important Analytical method to determine the sites of the 5-Mc in the genome is the bisulfite modification of the genome [58]. To understand

the role of DNA methylation in various disease states it is very important to quantify and detect the 5-Mc and bisulfite modification is considered as a very efficient method and a gold standard in doing so [59].

4.3.3. Principle of bisulfite conversion

It is based on the selective reactivity of sodium bisulfite with cytosine's and methylated cytosine's. The chemistry of cytosine deamination involves three steps namely, Sulfonation which involves addition of bisulfite to the 5, 6 bond of the cytosine in the presence of acidic pH, hydrolytic deamination which is an irreversible conversion of cytosine bisulfite derivative to Uracil bisulfite derivative aided by some free radical scavengers to prevent any oxidization. This is followed by the alkali desulfonation of the Uracil bisulfite derivative to Uracil in the presence of an alkali to decrease the pH and aid in desulfonation [60]. Bisulfite treatment deaminates cytosine to Uracil and leave 5-methylcytosine unchanged. One of the major advantage of this method is the integrity of the DNA is maintained and thus enable various techniques for amplifying or analyzing DNA to be performed on the bisulfite modified DNA, thereby can startup with low Concentrations of DNA [48] Figure 4.5.

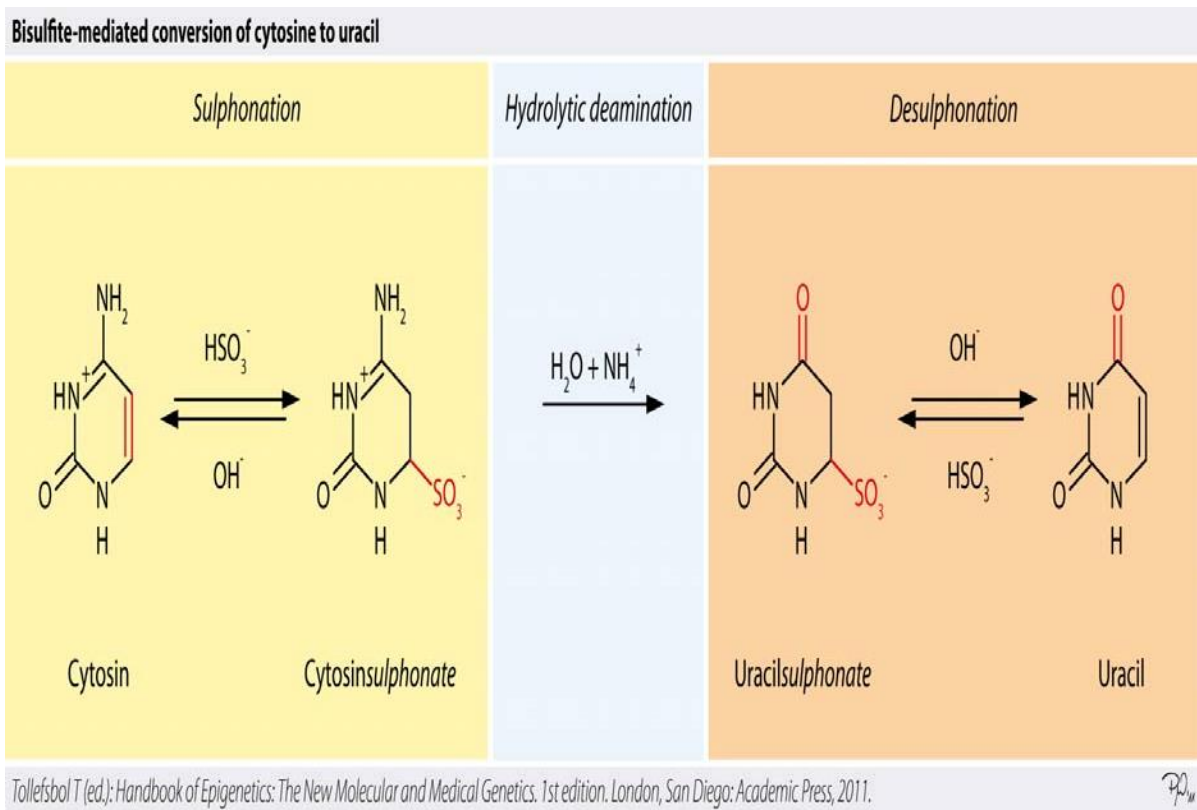


Figure 4.5, Bisulfite mediated conversion of cytosine to uracil [61]

The efficiency of the bisulfite conversion is dependent on the following errors like [62], failed conversion when unmethylated cytosine do not undergo deamination and as a result it appears methylated and thus gives an increased estimation of methylation and often it can be corrected by increasing the time of bisulfite treatment or by increasing the number of denaturation steps [63], by doing so can also prevent the renaturation of sample DNA. Inappropriate conversion is another important problem which occurs when methylated cytosine undergo deamination yielding thymine and they like Uracil pair up with adenine during PCR and are misinterpreted as unmethylated leading to underestimation of the methylation density [64]. Depurination during the time of conversion is another notable problem by failing of enzymatic reactions when using negligible amounts of DNA [65]. Degradation of template DNA is a very common side effect seen and it affects the detection limit of method but how much of DNA is lost during the reaction is not known. Usually, at a temperature of 95-degree Celsius DNA degrades rapidly and thus higher temperatures are preferred only if sufficient amount of DNA is available and it is always advisable to keep the incubation time as low as possible since it is evident that the longer the incubation time greater the degradation [64]. However, DNA methylation Analysis on the basis of bisulfite modification are performed on a larger spectrum and many methods of amplification enable a bisulfite converted DNA concentration of 50ng sufficient and thus enabled the design of various techniques based on bisulfite conversion.

4.3.4. Primer design

The primer design is a very important parameter and is essential in determining the methylation status of a gene promoter. Some of the important parameters to be taken care while designing a primer are, they should be at a length of 23-24 bp to achieve desired gene specific annealing [66]. Exact Annealing temperature of the primer has to be determined, because low stringency leads to mismatching and MSP depends on specific annealing to the DNA. Amplification of the unconverted CpG dinucleotide which is unmethylated may show greater methylation levels. Therefore, the primers should be designed to be specific to Bisulfite modified DNA and also contain few non CpG cytosines in the original template [48].

4.3.5. Applications of MSP

Various methods of MSP have been developed to increase the sensitivity of detection of MSP for the methylated DNA. Nested MSP is on such method which is more sensitive and allows detection of very small quantities of DNA. However, problems like renaturation, incomplete bisulfite conversion and lack of accurate information about the single strand are seen [67, 68]. Quantitative MSP is useful to quantify the number of methylated alleles. It is mainly advantageous as it does not involve steps like gel electrophoresis, enabling it's us as an important tool in fast screening [69].

It is very difficult to find out the exact timing of the methylation changes by MSP. To prevent these types of issues ISH have been used. It was used in combination with MSP and aid in the detection of methylated DNA on tissue slides [70]. It is used to examining

the progress of tumor, CpG island methylation and gene expression. HPLC is also used and various forms of HPLC like denaturing HPLC are used in combination with MSP and they detect methylation patterns based on the denaturation temperatures [66]. Ion Pair-Reverse phase HPLC was also used to distinguish between methylated and unmethylated with the help of deoxynucleotides [71]. Denaturing gradient gel electrophoresis is a method based on the thermal stability useful to detect the overall methylation differences and it is the first study to differentiate between methylated, hemi methylated and unmethylated sites [72, 73]. These are some of the methods which use MSP as a combination and try to increase the sensitivity of the MSP and aid in better detection between methylated and unmethylated DNA.

4.4. Biomarkers

A biomarker can be defined as, an indicator used to measure the normal biological function, pathogenicity and response to drug treatment [74]. A biomarker can either be secreted by the malignancy itself or as a physiological response to the presence of the cancer. A clinical end point usually determines the efficacy and the biomarkers are mainly used to form an intermediate endpoint and are thus used to monitor earlier stages of carcinogenesis [75]. Biomarkers are distinguished mainly into two types, Biomarkers which are used to determine the successful dosage levels required and those which are used to determine the progress of a disease [76]. Biomarkers in cancers have multidimensional role [77], they serve in screening studies and diagnosis. Biomarkers used in screening studies usually check the existence of any premalignant conditions or the exposure and in diagnostic role, they are mainly used to diagnosis the presence or

absence of cancer in a certain individual. They also constitute a role in the prevention trials of cancer and its treatment as they focus on various effects, like the side effects induced and they further focus on the toxicities rather than the desired result. The primary defect in Cancer is in the genomic DNA and alterations of DNA like translocation, mutations, aberrant DNA methylation play a major role in carcinogenesis and the biomarkers which can bring about these DNA alterations are used as potential tumor biomarkers. The use of DNA as a tumor marker has several advantages. When in comparison with proteins they can be amplified and therefore useful in the detection of minute quantities of the samples. High stability when compared to m-RNA and the proteins which aids its survival in adverse conditions for longer periods [78].

4.4.1. DNA methylation as a bio-marker

The aberrant methylation of the promoter regions of genes is seen both in early as well as advanced cancers and when DNA with these aberrantly methylated genes is released into the blood serves as an indicator for the earlier detection of the cancer. This ability to detect cancer in its earlier phases makes DNA Methylation an important marker over other markers which cannot detect cancer in its earlier phases of tumorigenesis [79]. The involvement of DNA methylation in both onset as well as progression of different cancers enables it to serve a dual role both as a diagnostic and a prognostic marker of disease. The main advantage of DNA methylation over mutations which can arise at any point in the gene is its site specificity thus increasing its sensitivity of detection.

4.4.2. DNA methylation as a diagnostic marker

Early diagnosis is very critical for the successful treatment of cancer. The traditional methods of diagnosis invasive as well as noninvasive like cytology, histopathology and immunohistochemistry are useful. The detection of cancer with minimal invasive procedure is always preferred. The changes in the marker should be specific so as to differentiate the level of malignancy and assess the premalignant conditions, thereby diagnosis the risk level of the progression of the malignancy. The sensitivity and the specificity of the DNA methylation marker depends on many factors in cancer diagnosis namely the type of body fluid used, type of cancer studied and techniques involved [6]. DNA methylation biomarkers can show few premalignant conditions which are not progressed to a level of detection detectable by other methods of detection by quantitative analysis of the levels before its progression to a fatal limit of malignancy. Some of the available diagnosis methods might show false results leading to further analysis and therefore costing time, money as well as psychological stress. This can be avoided by using DNA methylation markers in combination with other tests as a panel and therefore increasing the sensitivity and specificity of the screening procedures.

4.4.3. DNA methylation as a prognostic and predictive bio-marker

The genes which undergo methylation at any stage of the cancer progression act as potential prognostic markers whereas predictive biomarkers are the markers based upon the therapeutic results [80]. For determining the best therapeutic mode of treatment, patient's response to the administered chemotherapeutic agent and survival, thereby

allowing monitoring of various changes accordingly, these markers are utilized. Usually prognosis is carried on in absence of the adjuvant therapy.

Predictive markers are usually evaluated when measurable amount of disease is usually seen whereas prognostic effect is usually evaluated as a control without any systemic treatment. Prognostic factors are mainly utilized to differentiate patients based on the aggressive of the disease thereby treating them accordingly by avoiding the unnecessary side effects. However, it is still unclear in many reports whether the predictive or the prognostic impact has been observed.

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CHAPTER V

QUANTITATIVE METHYLATION ANALYSIS OF TUMOR ASSOCIATED GENES USING METHYLATION SPECIFIC PCR FOR THE DETECTION OF HEPATOCELLULAR CARCINOMA

5.1. Introduction

Hepatocellular carcinoma (HCC) represents a major international health problem as a result of its increasing incidence, with 700,000 new cases raised according to the records in 2012. The regions of the high incidence of HCC particularly in China, Thailand and Africa [1]. The survival rate of HCC is low due to its poor diagnosis and prognosis, high recurrence, and resistance to chemotherapy and radiotherapy [2]. Various epidemiological studies have shown that 70% of HCC cases in the United States and 75% of cases in developing countries are associated with HCV and HBV infection [3-5]. The major risk factors for

HCC are liver cirrhosis, hepatitis B and C virus, dietary aflatoxin exposure, obesity and excessive consumption of alcohol [6, 7]. However, the majority of HCC was detected in non-resectable advanced stages, which further prevents potential curative treatments.

Recent reports demonstrate that epigenetic inactivation of gene expression by aberrant methylation on CpG islands may be a fundamental contributor to carcinogenesis and cancer progression [8 -10]. Based on direct inactivation of tumor suppressor genes, DNA hypermethylation can also block transcription factors and silence DNA repair genes, resulting in the loss of downstream gene functions and the accumulation of genetic lesions [11]. For HCCs, a growing number of genes undergo hypermethylation in liver tissues [12-16]. Supporting the hypothesis that determination of methylation in specific genes may be useful for HCC diagnosis. A-fetoprotein (AFP) testing, the only approved screening test, has been used to detect HCC for many years, but its sensitivity and specificity are marginal [17]. Hence, there is an urgency to establish a better set of DNA methylation markers for early diagnosis and prognosis of HCC.

DNA methylation appears to be an emerging tumor biomarker and frequent promoter of methylation of various tumor suppressor genes [18-20]. Current testing assays utilize single DNA marker to detect HCC, but there is no single marker that is both sensitivity and specific enough to meet the HCC screening requirements. Recent studies demonstrated the advantages of multiple methylated genes in both tissue and plasma samples regarding diagnostic and prognostic information [21-23]. However, little was known about the value of DNA methylation analysis at multiple gene sites for the detection of HCC in various population.

The aim of this study was to identify a combination of methylated genes that was suitable for clearly well-differentiated between HCC and adjacent non-HCC, and to establish a user-friendly methylation-specific PCR (MSP) system to measure methylation status of these HCC gene markers. To achieve the aim, based on the publicly available database we found 160 methylated genes from 2000 to 2015 (Table 5.1). We selected five genes (GLOXD1, B4GALT1, CHFR, BLMH, miR-129-2) that have been reported to be aberrantly methylated by other groups. Using this information, we evaluated individually and the combination of the methylation status of these genes in detecting and diagnosing HCC.

Table 5.1, DNA hypermethylation genes list for HCC from 2000-01-01 to 2015-07-30

S.NO	Genes	Number of samples						Reference
		HCC	Adj HCC	HBV	HCV	LC	NC	
1.	3OST2	33/48	18/48					[26]
2.	14-3-3E							
3.	AKAP12	41/48	10/48					[34]
4.	APC	411/668	20/96	0/19	33/46	76/113	51/99	[36-40,15-18,72,78,21]
5.	ASS							
6.	B4GALT1	15/27	0/20					[25]
7.	BASP1							
8.	BCL6B	129/149	20/50				0/8	[40]
9.	BLMH	28/48	0/48					[29]
10.	BMP-6	39/60	30/60					[11]

11.	BTG3							
12.	CADM1	34/82	9/56				0/8	[12]
13.	CASP8	Methylated in both HCC and Adj non-Cancerous tissue						[13]
14.	CAV1							
15.	CCND2	68/161	10/98				14/25	[14-16]
16.	CDH1	138/428		0/19	7/38	8/41	32/133	[6, 18-20]
17.	CD82							
18.	CDO1	52/123		2/29		4/28	0/20	[21]
19.	CHFR	62/178	1/48				0/60	[1, 27, 28]
20.	CFTR	73/80	15/55					[16,20, 15]
21.	CIS	70/80	33/80					[28,76]
22.	COX-2	33/108	2/48	0/19	0/19	0/30		[26, 6]
23.	CSRP1	9/16						[22]

24.	DACH1	24/57	9/57				0/2	[23]
25.	DAPK	107/224		0/19	22/51	70/98	13/45	[4,6, 17, 21]
26.	DENND2D	69/92						[58]
27.	DKK1	64/216					0/75	[18]
28.	DKK3							
29.	DLC-1	61/216					0/75	[18]
30.	DLEC1							
31.	DLGAP5	168/220	22/88					[56]
32.	DNMT1	24/44				11/35		[31]
33.	DNMT2	8/44				4/35		[31]
34.	DNMT3A	30/44				14/35	7/35	[31]
35.	DNMT3B	17/44				9/35		[31]
36.	E2F1							
37.	ESR1	40/48						[62]

38.	EYA4							
39.	FAM83D	50/72	9/72					[29]
40.	FAM43B	24/40						[105]
41.	FBLN1							
42.	FHIT							
43.	GLOXD1	75/160	4/160				2/29	[24]
44.	GSTP1	322/545	22/97	0/19	0/13	32/93	7/85	[4, 6,16,19,20,72,78,21,121]
45.	GRASP	17/24						[32]
46.	GRS17							
47.	GPX3	46/60	0/60					[37]
48.	HACE1	18/27						[61]
49.	HAI-2/PB							
50.	HDPR1							
51.	HIC1	32/48	41/48					[26]

52.	HINT1	22/40	15/40					[113]
53.	HISTIH2A E							
54.	hMLH1	9/110	3/50	0/19	0/13	0/30		[6,121]
55.	HOXA9	23/30	16/30				1/29	[35]
56.	IGFBP-7	89/136		8/46			5/35	[59]
57.	IRAK3	102/160	23/160				1/29	[35]
58.	JNK1	17/31						[114]
59.	KL							
60.	KLK10							
61.	KLHL35							
62.	LIFR							
63.	LINE-1	37/75	52/75					[40]
64.	MAGE-A1							

65.	MAGE-A3							
66.	MAT1A							
67.	MGMT	81/323	5/50			14/79	17/77	[4, 20,78,21,121]
68.	MT1G	114/169	8/48	6/37			3/31	[44,45]
69.	MT1M	59/121		2/37			2/31	[44]
70.	MTSS1							
71.	MUC2	46/74	14/74					[64]
72.	NBS1	6/64						[55]
73.	NORE1B							
74.	NQO1							
75.	NR2E1							
76.	O6MGMT	84/208			20/100	60/108	4/100	[53]
77.	OPMCL	35/50	32/50					[121]
78.	OXGR1							

79.	p14ARF	178/445	23/80	2/24	33/138	61/119	46/158	[19,20,53,57,116,121]
80.	p151NK4B	196/389	25/50	6/76	20/100	36/108	4/163	[17,34,53,57,77,121]
81.	P16	510/856	66/344	5/97	23/68	23/121	26/205	[4,6,14,19,20,34,52,57,72,77,78,109,21, 111,121]
82.	P21	22/50						[34]
83.	P27	1/50						[34]
84.	P3000							
85.	P53							
86.	P73	170/289	0/50		28/138	60/108	4/113	[19,53,121]
87.	PAX							
88.	PCDH10	38/50	20/50					[101]
89.	PER3							
90.	PENK							
91.	PRDM2							

92.	PRL-1							
93.	PTEN							
94.	RASSF1A	873/1158	30/144	16/15 7	25/53	50/181	8/267	[3,6,8,16,19,34,38,50,68-78]
95.	RASSF2A	31/45	12/30					[112]
96.	RASSF10	57/69					0/20	[30]
97.	RAR-b	15/50	3/50					[121]
98.	RB	18/64		6/24				[57]
99.	RECK							
100.	RELN	18/48						[108]
101.	RIZ1	111/189	3/48				1/17	[4,78,110,115]
102.	RUNX-3	337/674	32/386				1/100	[4,16,18,39,72]
103.	SAMSN1	124/144						[36]
104.	SDPYA							

105.	SFRP1	215/458	9/47	3/21		9/42	2/115	[4,18,72,102,119, 120]
106.	SFRP2	54/96		4/21		9/42	2/32	[4, 121]
107.	SFRP3	36/60		6/37		9/23	0/37	[49]
108.	SFRP4	3/54		0/21		0/42	0/15	[120]
109.	SFRP5	33/96		7/42		7/42	2/32	[4, 120]
110.	SHP1	70/80	73/80					[76]
111.	SIP1	17/23						[84]
112.	SKY							
113.	SLIT2	45/48	39/48					[26]
114.	SOCS1	372/624	56/130				0/17	[4,19,76,99,118,121]
115.	SOCS2	68/80	20/80					[76]
116.	SOX1	31/54		3/21		14/42	0/15	[66]
117.	SOX17							
118.	SPARC	45/60	7/60					[100]

119.	SPINT2	64/80			32/50		20/50	[16,33]
120.	SRD5A2							
121.	SSBP2	14/27	6/18					[8]
122.	Survivin							
123.	SYK	34/65						[19]
124.	TFPI2	20/43		4/26			5/26	[98]
125.	TGR5	77/160		12/88			2/45	[51]
126.	TIP30							
127.	TIF1Y							
128.	TIG1	48/91	17/91					[60]
129.	TSPYL5	18/24						[31]
130.	TUSC1	29/94	2/94					[54]
131.	UCHL1	12/27						[118]
132.	UNC5C	11/42						[67]

133.	Vimentin	24/43						[105]
134.	WIF-1	153/321	39/105					[18]
135.	WT1							
136.	XAF1	43/65						[19]
137.	XPO4	28/44		9/34		18/38	3/17	[48]
138.	ZAR1	67/88						[63]
139.	ZHX2							
140.	ZIC1	72/132	19/132					[46]
141.	miR-1	18/40						[43]
142.	miR-9-1	30/80						[57,73]
143.	MiR-9-2	20/80						[57,73]
144.	miR-10a							
145.	miR-34a	33/83	22/43					[57, 68]
146.	miR-34b/c	34/43	23/43					[68]

147.	miR-124-1	8/40						[57]
148.	miR-124-2	5/40						[57]
149.	miR-124-3	17/40						[57]
150.	miR-1247	15/40						[57]
151.	miR-125b							
152.	miR-129-2	127/198	6/75			1/8	0/12	[55-57]
153.	miR-132							
154.	miR-148a	3/40						[57]
155.	miR-203	0/40						[37]
156.	miR-320							
157.	miR-335	18/20	25/32					[86]
158.	miR-596	11/40						[57]
159.	miR-663							
160.	miR-9							

5.1.1. MiR-129-2 as a biomarker

Micro RNA a group of small noncoding RNA with a length of ~22 nucleotides, participates in numerous biological and pathological processes, including development regulation, cell proliferation, cardiogenesis, lymphocyte development, adipocyte differentiation, and carcinogenesis, through transcriptional or translational repression. MiR-129-2 gene is located in a canonical CpG island on chromosome 11, which was found to be frequently hypermethylated in endometrial cancer and gastric cancer. Tumor-specific down-regulation of subsets of miRNAs has been described in the initiation and progression of HCC.

Based on evidence has emerged that epigenetic mechanisms play a crucial role in the down-regulation of tumor suppressing miRNAs, and contribute to malignant transformation during hepatocarcinogenesis. Based on publicly available data source the overall specificity and sensitivity of MiR-129-2 were 92.63% and 64.14%. Based on the data we selected MiR-129-2 as a biomarker in our research [55-57].

5.1.2. CHFR as a biomarker

Checkpoint with Forkhead and Ring Finger Domains, E3 Ubiquitin Protein Ligase (CHFR) is a protein coding gene. It is located on chromosome 12 from 132, 822, 187 bp to 132, 956, 304 bp. primary human tumors and mammalian cell culture models indicate that CHFR may function as a potent tumor suppressor. CHFR functions as part of an early G2/M checkpoint, more specifically in antiphase. Antiphase refers to late G2 when chromosome condensation starts. This early mitotic checkpoint causes a delay in chromosome condensation in response to mitotic stresses. The human CHFR gene was originally identified during a search for novel cell cycle checkpoint proteins that have

fork-head associated domains. Initial analysis indicated that the CHFR-associated G2/M checkpoint was inactivated in a subset of cancers as demonstrated by high mitotic indices [a high percentage of cells that have condensed chromosomes] in response to exposure to the microtubule poison, nocodazole, due to lack of CHFR expression or CHFR mutations in various cancers. Many other studies showed promoter hypermethylation leading to low/no expression of CHFR.

Aberrant methylation was detected in 22 of 65 (35%) primary hepatocellular carcinomas (HCC), compared to noncancerous liver cells [27]. Also, methylation of CHFR was found to be significantly correlated with advanced disease stage ($p=0.037$) and an infiltrated growth pattern ($p=0.047$). In another study with 70 HCC samples, methylation frequency of CHFR was 43% (30 out of 70) [28]. Based on publicly available data source the overall specificity and sensitivity of CHFR were 99.19% and 34.83%. based on these values we selected CHFR as a biomarker for our research [1,27, 28].

5.1.3. BLMH as a biomarker

Bleomycin hydrolase (BLMH) is cytoplasmic cysteine peptidase that is highly conserved through evolution. It is located on chromosome 17 from 30, 248, 195 bp to 30, 292, 166 bp. However, the only activity of the enzyme is metabolic inactivation of the glycopeptide bleomycin (BLM), an essential component of combination chemotherapy regimens for cancer. The protein contains the signature active site residues of the cysteine protease papain super family.

Based on “Triple-combination array” experiment many genes showed differential expression between normal tissue and tumor tissue. Analyzing the data derived from the triple-combination array, the bleomycin hydrolase gene was given highest priority of all candidate genes because bleomycin is widely used as anti-cancer drugs. Based on publicly available data source the specificity and sensitivity of BLMH were 100% and 58.33%. Based on these results we selected BLMH is a biomarker for our research.

5.1.4. B4GALT1 as a biomarker

β -1, 4-galactosyltransferase (B4GALT1) is a protein coding gene. It is located on chromosome 9p13. This gene is unique among the beta4GalT genes because it encodes an enzyme that participates both in glycoconjugate and lactose biosynthesis. It is ubiquitously expressed but at very low levels in the fetal and adult brain. This gene encodes type II membrane-bound glycoprotein, named β 1, 4-Gal-T1, which can transfer galactose in a β -1, 4 linkages to acceptor sugars. B4GALT1 is reported to be involved in the synthesis of selectin ligands, the skin wound-healing process, and the inflammation reaction.

The activation of this pathways plays an important role in human hepatocellular carcinoma. Based on the previously published papers the sensitivity and specificity of B4GALT1 were 55.55% and 100%. We selected as a biomarker for our research [25].

5.1.5. GLOXD1 as a biomarker

4-Hydroxyphenylpyruvate Dioxygenase Like (GLOXD1) as a protein coding gene. It is located on the chromosome 1 from 45, 326, 873 bp to 45, 328, 675 bp. It is an important enzyme in the catabolic pathway of tyrosine in the liver.

Based on the publicly available data the specificity and sensitivity of GLOXD1 were 96.74% and 46.87% [24]. Based on these results we selected as a biomarker for our research.

5.2. Materials and methods

5.2.1. Collection of clinical tissue specimens

We analyzed 80 tissue samples, consisting HCC and paired non-HCC liver tissues as control (Including 23 patients with HBV, 11 with cirrhosis, 33 with both HBV and cirrhosis and 13 without HBV and cirrhosis) in accordance with the institutional ethical guidelines. All patients were subjected to pathological diagnosis and classification for the different stages. For all patients, liver tissue samples were collected from cancerous and the adjacent non-cancerous surgical margin. All of these tissues were stored as formalin-fixed paraffin-embedded (FFPE) samples. The clinicopathological data of the patients at initial diagnosis were listed in Table 5.2.

Table 5.2, Clinicopathological parameters of 80 patients used in the independent validation study by qMSP

Clinicopathologic parameters	Cases (N = 80), n (%)
Age (Years)	
Range	32-82
Mean	52±9.75
Gender	
Male	63 (78.75)
Female	17 (21.25)
Liver Cirrhosis	
Yes	42 (52.50)
No	38 (47.50)
HBV	
Yes	54 (67.50)
No	26 (32.50)
TNM stage	
I	1 (1.25)
II	38 (47.50)
III	25 (31.25)
I-II	2 (2.70)
II-III	14 (17.50)
AFP	
≤ 200	24 (30.00)
>200	34 (42.50)
Unknown	22 (27.50)
Tumor size	
< 5 cm	48 (60.00)
≥ 5 cm	32 (40.00)

5.2.2. DNA extraction and purification protocol

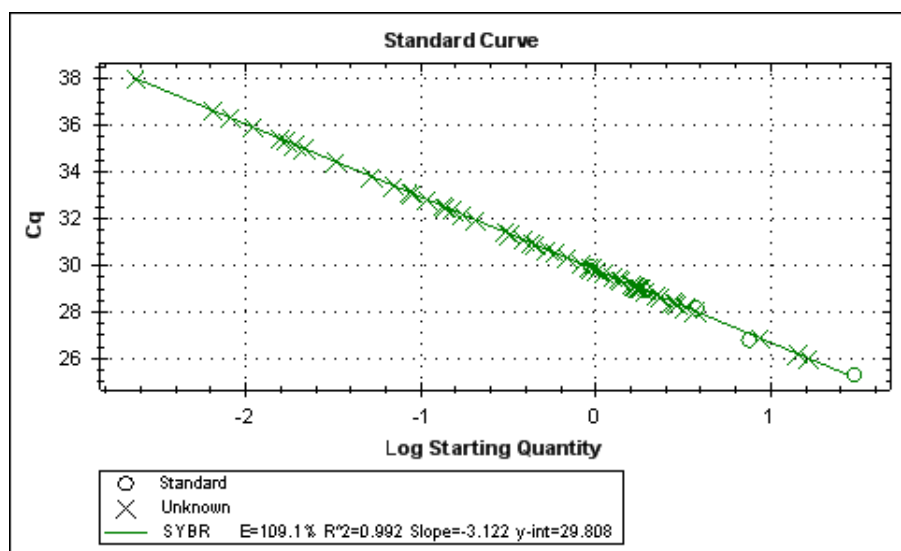
- Cut the tissue with microtome blade
- Wash the tissue with Xylene [incubate the tissue sample for 15 to 20min]. wash the tissue with 100% ethanol, 80% ethanol, two times with PBS
- Add the 10% SDS kept at 37°C for 1hr then add the Proteinase K and kept for overnight at 37 °C
- Add two volumes of Phenol Chloroform isoamyl alcohol [25:24:1], followed by the centrifugation at 10,000 rpm for 5min
- Collect the supernatant liquid into clean and fresh centrifuge tube
- Wash the solution with chloroform and collect the aqueous layer
- Add 1/10 volume of 3M sodium acetate and two volumes of isopropanol
- Keep the solution at -20 °C for overnight.
- Centrifuge the solution for 15min at 15,000 g
- Discard the liquid and wash the pellet with ice chilled 70% ethanol
- Air dry the pellet for 10 to 15min
- Add the 0.1mM of Tris-EDTA

Once we extract the DNA we calculate the DNA concentration with the help of UV-Vis and PCR. By using UV-Vis we measure the absorbance of DNA at A_{260} nm. DNA concentration is estimated by measuring the absorbance at A_{260} nm, adjusting the A_{260} measurement for turbidity (measured by absorbance at 320nm), multiplying by the dilution factor, and using the relationship that an A_{260} of 1.0 = 50µg/ml pure dsDNA.

$$\text{Concentration (}\mu\text{g/ml)} = (A_{260} \text{ reading} - A_{320} \text{ reading}) \times \text{dilution factor} \times 50\mu\text{g/ml}$$

By using PCR, we plot a standard calibration curve with the help of serial dilution of standard genomic DNA along with the samples. With the help of slope, Y-intercept and Ct values of the sample we calculate the unknown DNA concentration (Fig: 5.1). As we can see in Table 5.3, PCR calculated concentrations are less than the UV-Vis calculated concentrations. because in the UV it reads all the DNA present in the sample but in PCR with the help of β -actin primer amplified DNA only is read and gives the Ct value. Based on that Ct value we can get the accurate DNA concentration. For our research, we use PCR calculated DNA concentration.

(A)



(B)

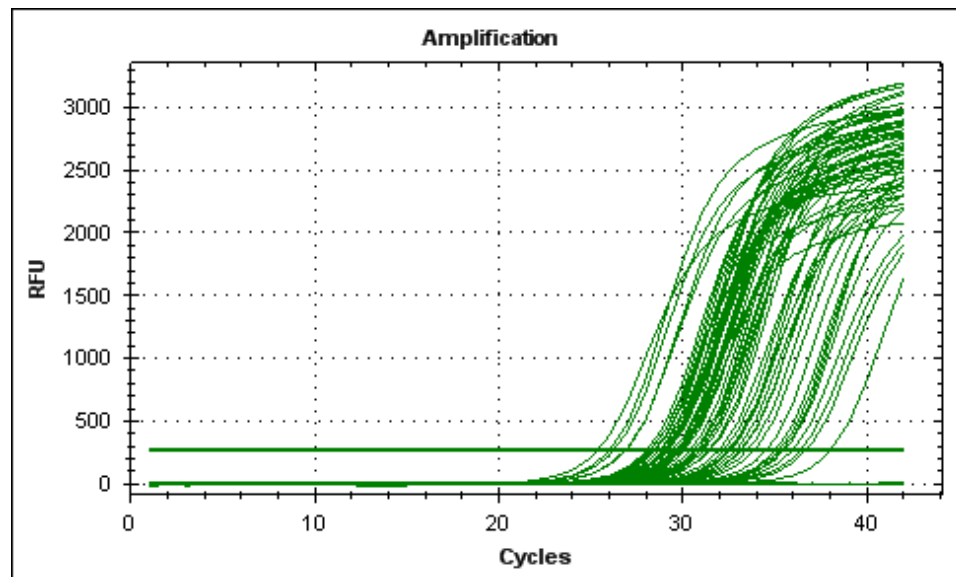


Figure 5.1, (A) Standard β -Actin curve. (B) Amplification curve for FFPE samples.

Table 5.3, FFPE tissue DNA samples concentrations

S.No	Normal sample	UV ng/mL	PCR concentration	Cancer sample	UV ng/mL	PCR concentration
1	01-13	695.5	300	01-13C	162.05	80
2	03-18	302.55	200	03-18C	422.85	280
3	03-17	343.85	220	03-17C	277.9	180
4	03-19	436.75	180	03-19C	437.25	90
5	03-16	533	315.4	03-16C	485.75	201.5
6	03-18	290.45	90.5	03-18C	529.5	333.9
7	02-08	597	287.1	02-08C	319.6	115.03
8	06-15	90.25	11.03	06-15C	0.75	24.5
9	04-29	111.35	27.36	04-29C	110	25.78
10	05-10	107.3	27.24	05-10C	63.4	16.74
11	05-08	132.45	19.15	05-08C	305.3	180.4
12	04-07	274.75	154.03	04-07C	194.85	32.9
13	07-23	449.4	265.1	07-23C	224.9	47.29
14	05-33	254.95	99.9	05-33C	334	29.98
15	04-08	440	116.74	04-08C	384.3	257.54
16	02-07	1043.5	334.5	02-07C	476.75	40.78
17	04-12	490.35	196.04	04-12C	1273	540.79
18	01-44	551.5	180.5	01-44C	660.5	136.81
19	03-42	466.4	277.68	03-42C	990	476.81
20	03-35	472.25	138.46	03-35C	773.5	41.39

5.2.3 Reagent preparation

5.2.3.1 Preparation of CT-conversion Reagent

The CT conversion supplied is a solid mixture and 900µL water, 300 µl M-Dilution buffer and 50µl M-dissolving buffer to a tube of CT conversion Reagent. It is thoroughly mixed with frequent vortexing for 10 minutes. It can be stored at -20 °C. It can also be prepared manually, Table 5.4. Light should be avoided as it is very sensitive and best if used immediately after preparation.

Table 5.4, CT-conversion reagent

Reagent	Composition	Quantity 96 samples
Sodium metabisulphite	Na ₂ S ₂ O ₅	5.8g
M-Dilution buffer	2MNaOH	2880μL
M-Dissolving buffer	50%dimethyl formamide	480μl
water	H ₂ O	1200μl

5.2.3.2 Preparation of M-wash buffer

24ml of 100% ethanol to the 6ml M-wash buffer or 96ml of 100% ethanol to the 24ml M- wash buffer concentrate should be added before use.

5.2.4 Protocol for bisulfite conversion

prior to sodium bisulfite conversion, DNA concentration was quantified by RT-PCR amplification. Standard unmethylated and methylated genomic DNA was purchased with CpGenome DNA modification kit (Millipore, Billerica, MA) for establishing a standard curve. Different concentration of standard DNA was obtained by serial dilution and then amplified with the β -actin (ACTB) primer: 5' GGCGGCACCACCATGTACCCT 3' and 5' AGGGGCCGGACTCGTCATACT 3'. Sample DNA concentration was calculated utilizing the standard calibration curve of cycle numbers and log concentration. Based on these results, the final genomic DNA concentrations of all samples were normalized within the same concentration through dilution adjustment.

1. 130 μ l of the CT conversion reagent prepared is added to 20 μ l of the DNA sample in a PCR Tube. The amount of the input DNA can be from 500pg-2 μ g. However, for optimum concentrations, 200-500ng is preferred.
2. If the DNA sample is less than 20 μ l, the difference can be made up by water.
3. 2 or 3 μ l of Salmon DNA is added to the sample.

4. Place the sample tube in the thermo cycler and reaction proceeds in following steps

98 °C for 10min

64 °C for 150min

4 °C storage up to 20 hours

5. The whole process for purification consists of binding of sample, desulfonation, binding buffer wash, ethanol wash, and elution.
6. 600µl of the M-binding buffer is added to the zymo spin IC column and column is placed into a collection tube of capacity 800µl.
7. The sample is added to the column and is inverted several times. It is centrifuged at full speed for 30s and flow through is discarded.
8. 200µl of the M-desulfonation buffer is added to the column and left a room temperature for 15-20 min. After the incubation, it is centrifuged for 30s.
9. 200µL of wash buffer is added twice and centrifuge after each for 30s.
10. At the end, the column is placed into a 1.5ml micro centrifuge tube and M-elution buffer (10Mm of Tris buffer) is added. It is centrifuged for 30s and DNA is eluted DNA can be used immediately or stored at -20°C for further use. For longer time it should be stored below -70 °C

5.2.5. Quantitative methylation-specific PCR

The quantitative methylation analysis was carried out on Bio-Rad CFX96 PCR Instrument. The initial concentration of the primer is 100µg. It is available in dry form. It is re- suspended by dissolving in the appropriate amount of water. It is then diluted to 5µmol/L. HRM master mix contains, DNA polymerases, SYBR green dye, optimized concentration of Q-solution NTPs and MgCl₂. Each 10 µl reactions consisted of 4 µl Type-it® HRM master mix, 4 µl of 5 µmol/L of both forward and reverse primers and 1µl of bisulfite-modified (EZ DNA Methylation-Gold kit) genomic DNA samples. The amplification condition was the following: 95 °C denaturing for 10 min, 42 cycles of 95 °C for 45 s, gene specific annealing temperature [Table 5.5] for 30 S, and extension at 72 °C for 45 s. A melting curve was performed to conform the specificity of the PCR products. Methylation percentage (MP) was calculated using the following equation:

$$MP = \left(\frac{\text{Before qMSP input amount of DNA}}{\text{after qMSP Calculated amount of DNA}} \right) \times 100$$

where after qMSP calculated DNA was getting by plotting standard curve by using methylated DNA reference.

Table 5.5, Primer sequences for quantitative methylation specific polymerase chain reaction

Gene	Forward	Reverse	Annealing Temp [°C]
B4GALT1	5'- TAGGAAACGGGTTTCGACG -3'	5'- CCGTCCACTTTCTTTACCG -3'	58
BLMH	5'- AGAAGGCGTCGGCGTCGTTT -3'	5'- TCAACGCCGTCGAAACTAACC -3'	60
CHFR	5'- TTTCGTGATTCGTAGGCGAC -3'	5'- GCGATTAACTAACGACGACG -3'	58
GLOXD1	5'- AGGATGTGATTAGGCGTGAGGTTC -3'	5'- AAAAAAACGAAACCCGTA ACTCCG-3'	62
miR-129-2	5'- TTAGTTTGTTTCGGTTTTAGGGTTC -3'	5'- CTAAATAACTACCGTCTTCTCGACG -3'	64

5.2.6. Specificity and sensitivity of MSP

The specificity of the MSP assay was evaluated by detecting the bisulfite-modified standard unmethylated DNA and Methylated DNA through high-resolution-melting (HRM) analysis repeatedly. The melting temperatures for each gene were recorded as reference for the patient sample analysis. Sensitivity of MSP was determined by mixing bisulfite-modified standard methylated DNA (1%) with standard unmethylated DNA (99%) together.

5.2.7. Statistical analysis

All statistical analysis was performed with the use of the IBM SPSS 23.0 software for windows (studentdiscounts.com). The difference of DNA methylation status between different groups was analyzed using the student t-test. Optimum cutoff values for the five genes were separately determined by maximizing both specificity and sensitivity for the detection of HCC. Receiver-operating characteristics (ROC) curves were established to determine a threshold value in distinguishing HCC from adjacent non-tumor and to determine the area under the curve (AUC). A p value of less than 0.001 was considered statistically significant.

5.3. Results

5.3.1. Specificity and sensitivity of MSP method

The specificity of the MSP method on 5 genes was evaluated using standard unmethylated DNA as negative control and methylated DNA as positive control to ensure

the completion of bisulfite conversion. In addition, two blank samples, one from the blank control added before the bisulfite conversion and the other added before PCR reaction, were used to account for false positive. As shown in Fig. 5.2, this highly specific test on the MiR-129-2 gene was illustrated in the PCR amplification and the subsequent melting curve analysis for standard methylated DNA and unmethylated DNA as well as a blank control. The selected primer and optimized PCR conditions ensured that only methylated DNA after bisulfite conversion could be amplified for all genes. The specificity of these MSP methods is high for all 5 genes.

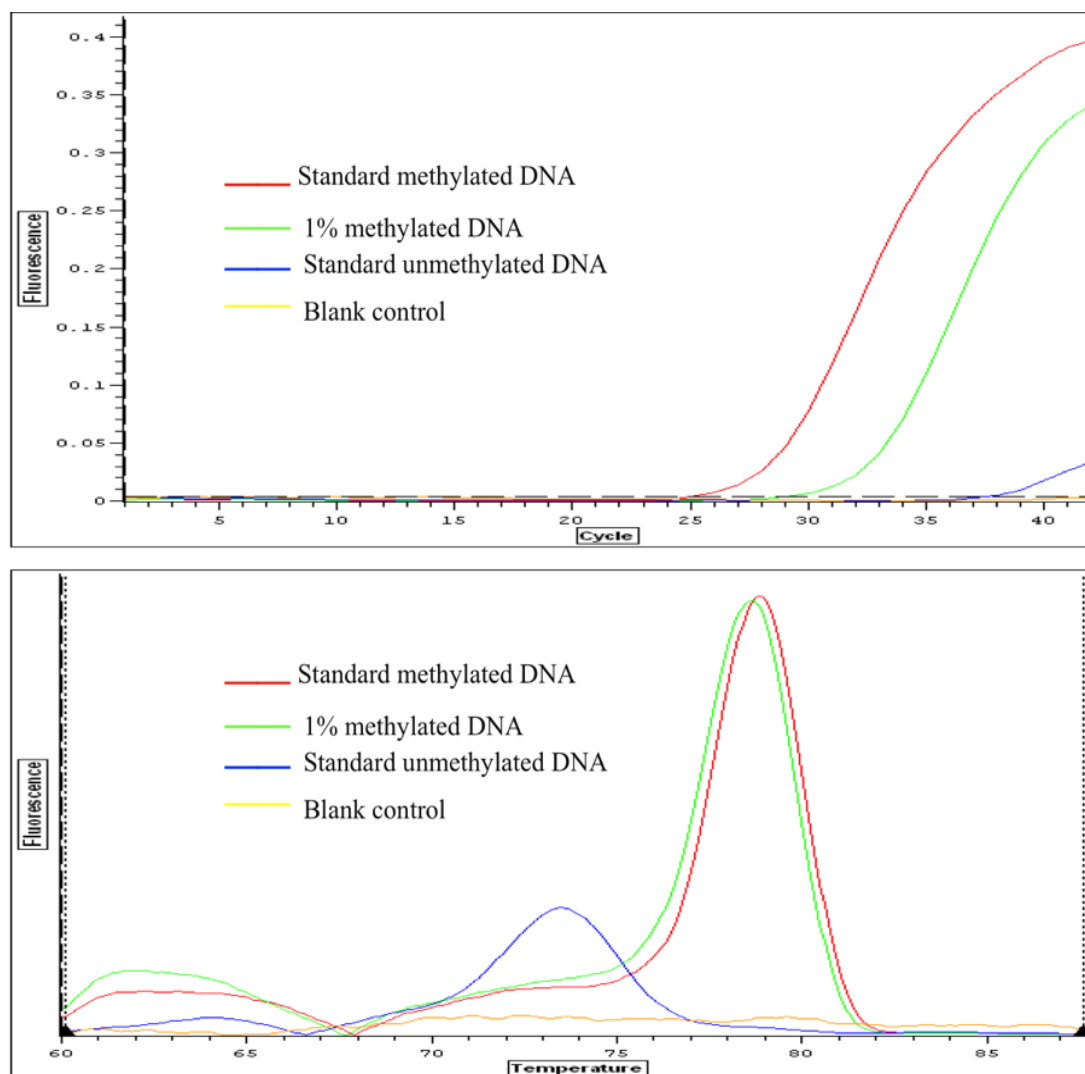


Figure 5.2, The amplification results of standard methylated DNA, 1% methylated DNA, standard unmethylated DNA as negative control, and blank control are shown in RT-PCR (A) and melting curve analysis (B).

The sensitivity of the MSP methods was assessed by mixing 1% standard methylated DNA and 99% standard unmethylated DNA together. Repeated amplification of the mixed sample were performed by RT-PCR. For all 5 genes, the MSP method could detect 1% methylation to ensure enough sensitivity of tests (Fig. 5.2).

During the patient sample MSP analysis, control samples including negative and positive control, and two blank sample, as well as 1% standard methylated DNA were amplified on the sample plate with patient samples. These controls ensured the completion of bisulfite reaction and the reliability of the results.

5.3.2. Quantitative methylation analysis

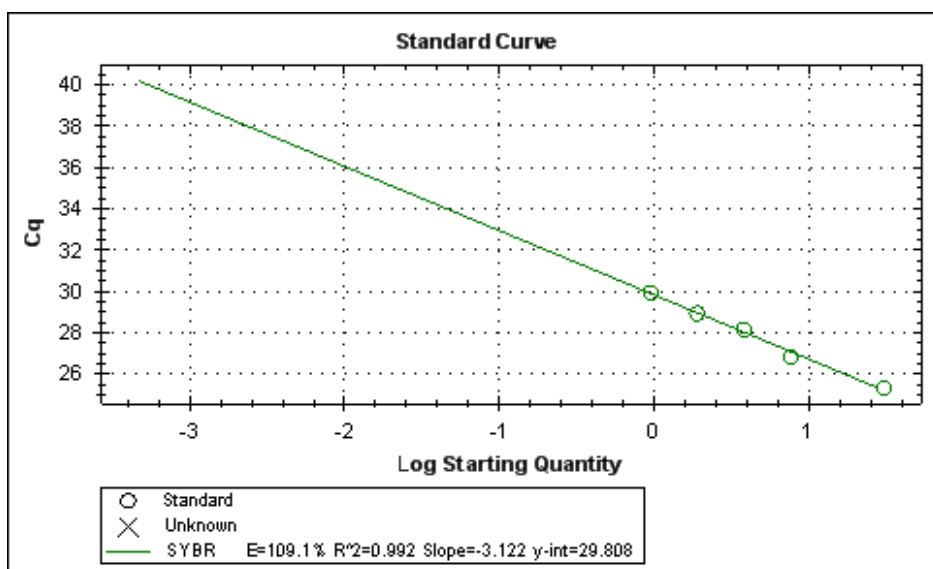
Although the promoter methylation on of B4GALT1, BLMH, CHFR, GLOXD1, and MiR-129-2, were high in HCC patient samples, the MSP analysis cannot differentiate the non-cancerous and cancerous tissue with the assistance of the melting curves. To obtain the quantitative information, we further performed RT-PCR methylation analysis of these three genes on 80 pairs of matched samples. The quantitative results were expressed as methylation percentage, which was determined by the following equation:

$$Methylation\% = \frac{Q_M}{Q_{Total}} \times 100\%$$

Q_M and Q_{Total} were the reference methylated and total DNA quantity, respectively. As shown in Figure 5.3, Standard curves were established to determine Q_M . Q_{Total} means total amount of DNA input in RT-PCR.

As summarized in Table 5.6, the quantitative results in 80 pairs of matched HCC tissues showed higher methylation percentage on genes B4GALT1, BLMH, CHFR, GLOXD1, and MiR-129-2 for cancerous samples comparing with the non-cancerous ones. The methylation percentage of cancerous tissue was high than in individual tissues.

A.



B.

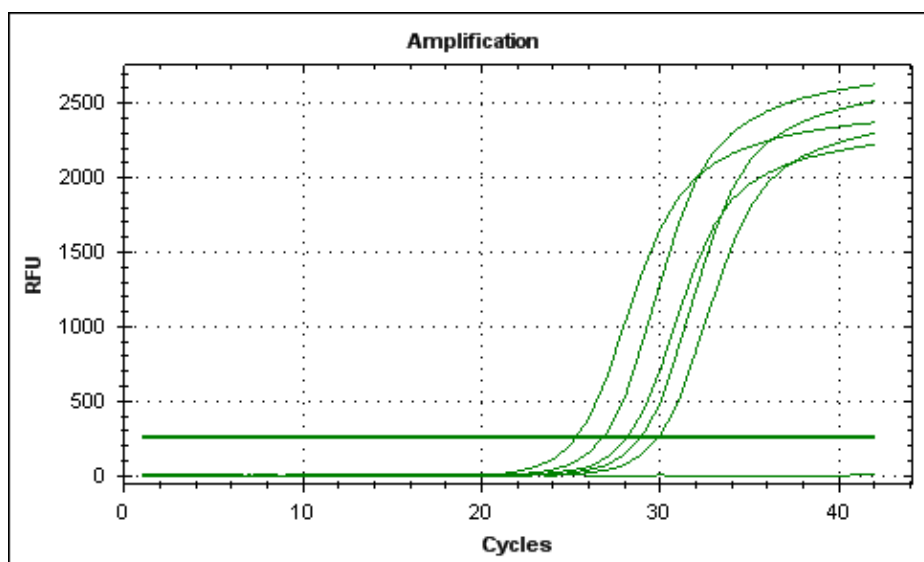


Figure 5.3, Standard curve constructed for quantifying methylated DNA for gene MiR-129-2 (A) and amplification curve for the MiR-129-2 standards (B)

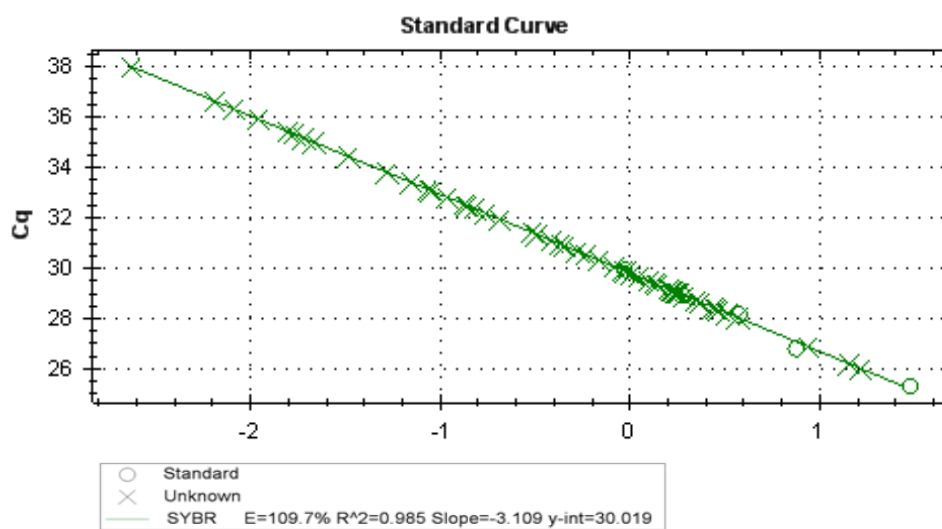
Table 5.6, The methylation percentage of 5 genes and combination of 5 genes on 80 pair of HCC cancerous/non-cancerous samples

Gene	HCC Cancerous Samples (Average)	Non-Cancerous Samples (Average)	N
B4GALT1	41.26	4.16	58
BLMH	59.78	2.71	43
CHFR	45.83	3.47	45
GLOXD1	59.72	7.26	50
MiR-129-2	54.41	3.48	60
Combination of 5 Genes	70.88	3.29	79

5.3.3. Gene-specific promoter methylation analysis

In epigenetic studies of HCC, the aberrant promoter methylation of B4GALT1, BLMH, CHFR, GLOXD1, and MiR-129-2 was frequently reported in previous studies [1, 24-30]. We checked the status of promoter hypermethylation for five genes in 80 liver tissue samples including 23 with HBV, 11 with cirrhosis and 13 without HBV and cirrhosis and for further validation using MSP. The AUC for the individual gene in discriminating HCC from adjacent non-HCC was moderate (GLOXD1: 0.695, B4GALT1: 0.721, CHFR: 0.672, BLMH: 0.649 and miR-129-2: 0.773). The methylation status was considered to be positive. All five genes exhibited higher methylation frequencies in HCC than adjacent non-HCC ($p < 0.001$), and the combination analysis resulted in an increased AUC of 0.975 with 93.7% of sensitivity and 100% of specificity, 100% PPV, and 94.1% NPV, respectively (Table 5.7 and Fig. 5.5). By adjusting the individual cut-off value of all genes (37.05% of GLOXD1, 31.05% of B4GALT1, 20.17% of CHFR, 30.85% of BLMH and 31.39% of miR-129-2). All samples were examined in this study had methylation in at least one gene.

(A)



(B)

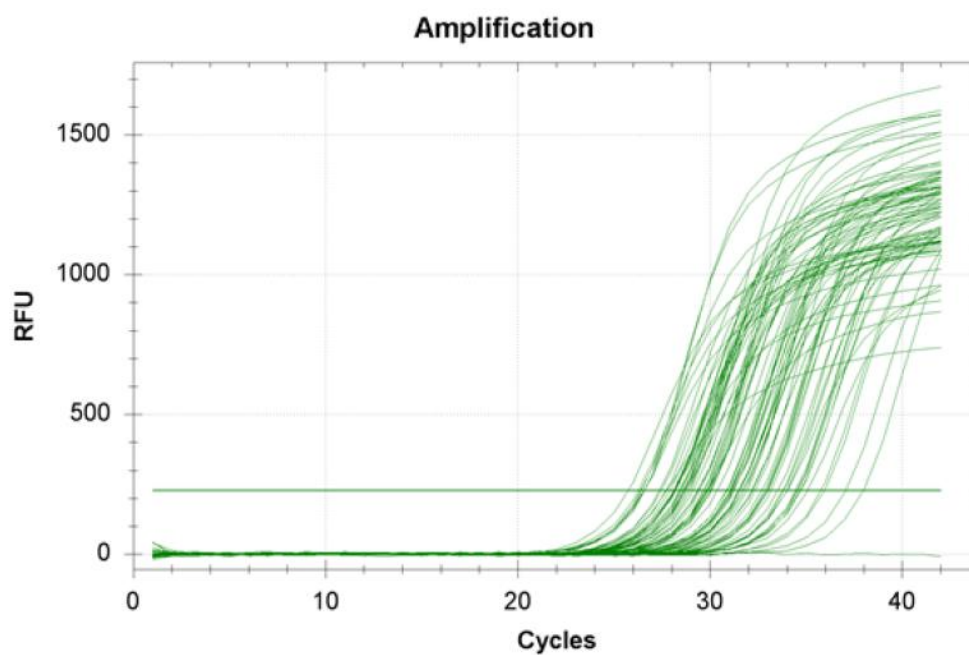


Figure 5.4, Standard curve constructed for quantifying methylated DNA for combination of 5 genes (A) and amplification curve for the combination of 5 genes (B)

Table 5.7, Diagnostic ability of five methylated genes based on qMSP analysis

Genes	AUC [95%CI]	Cut-off value [%]	Sensitivity [%]	Specificity [%]	PPV [%]	NPV [%]
GLOXD1	0.695	37.1	51.2	98.7	98.8	67.2
B4GALT1	0.721	31.1	47.5	100	100	65.6
CHFR	0.672	20.2	50	100	100	66.7
BLHM	0.648	30.8	48.7	100	100	66.1
miR-129-2	0.772	31.4	62.5	100	100	72.7
GLOXD1, B4GALT1, CHFR, BLHM and miR-129-2	0.975		93.7	100	100	94.1

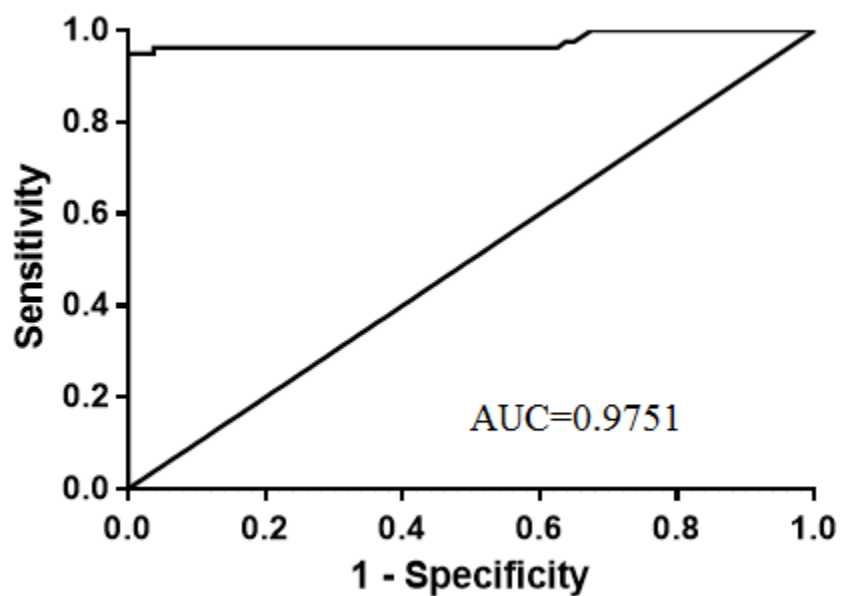


Figure. 5.5, Receiver-operating characteristics (ROC) curve for the combined analysis of DNA methylation levels (GLOXD1, B4GALT1, CHFR, BLMH, and miR-129-2) in 80 paired HCC and adjacent non-tumorous tissues. AUC: area under the curve.

5.3.4. Methylation levels of multigene in HCC

At first, the methylation status of these five genes were evaluated using MSP in 80 paired HCC and adjacent NT tissues and revealed that the MPs of all genes were higher in tumor tissues compared with adjacent NT samples (student t-test, $p < 0.0011$). The MPs of these five genes for all 80 samples are shown in Fig. 5.5. We found the clear differentiation between HCC and NT (Fig. 5.7. $P < 0.0001$). The diagnostic ability of these five methylated genes and combination was evaluated using ROC curve analysis. The AUC for each individual gene was low to moderate (range: 0.649 to 0.773, Fig. 5.8). However, the combination analysis of these five genes resulted in an increased AUC of 0.975 with 93.7% sensitivity, 100% specificity, 100% positive predictive value (PPV), and 94.1% negative predictive value (NVP) in discriminating HCC from adjacent NT tissues.

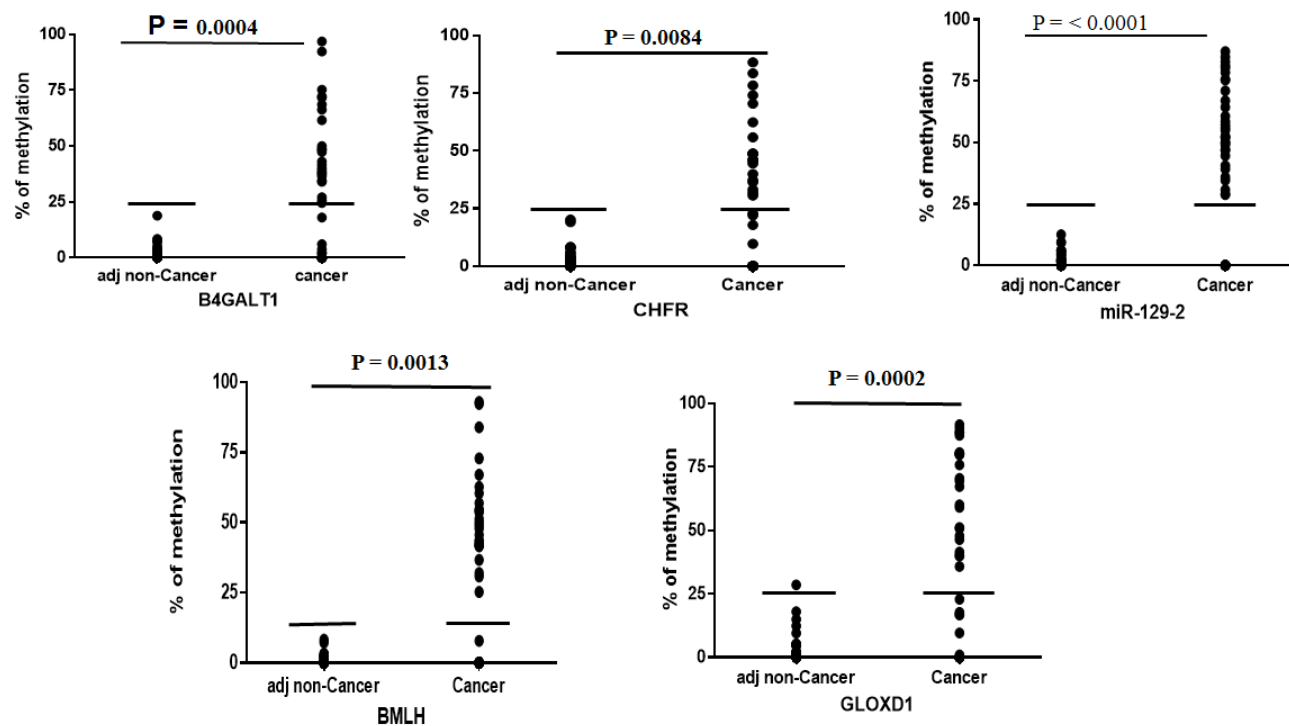


Figure. 5.6, Quantitative methylation results of qMSP on hepatocellular carcinoma and adjacent non-tumorous tissues The line represents the cut-off value. Mann-Whitney U test was used to determine statistical significance.

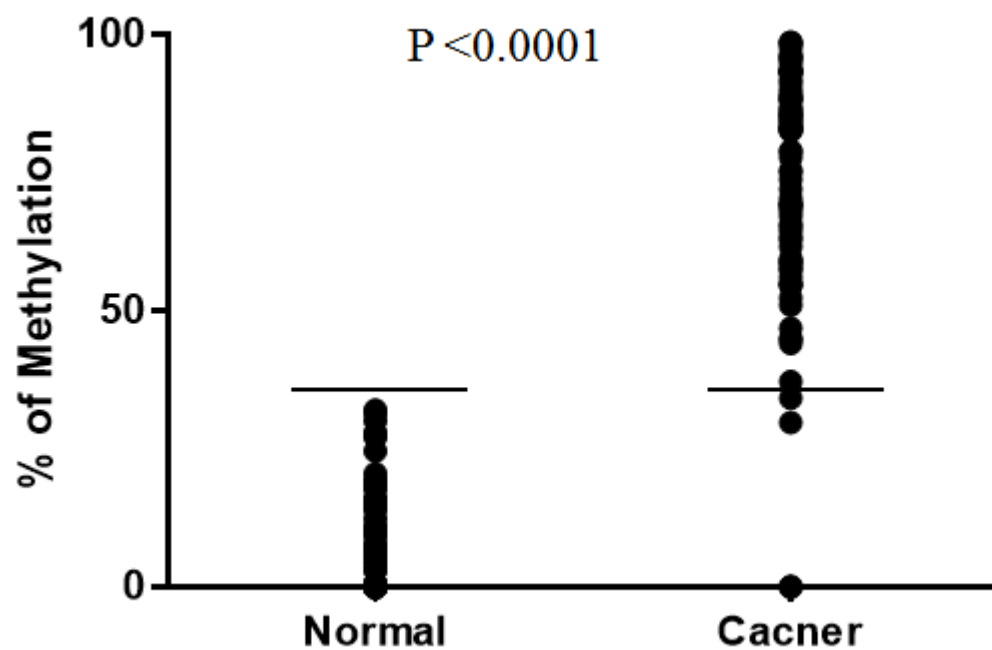


Figure. 5.7, Quantitative methylation results of qMSP for combined genes on hepatocellular carcinoma and adjacent non-tumorous tissues.

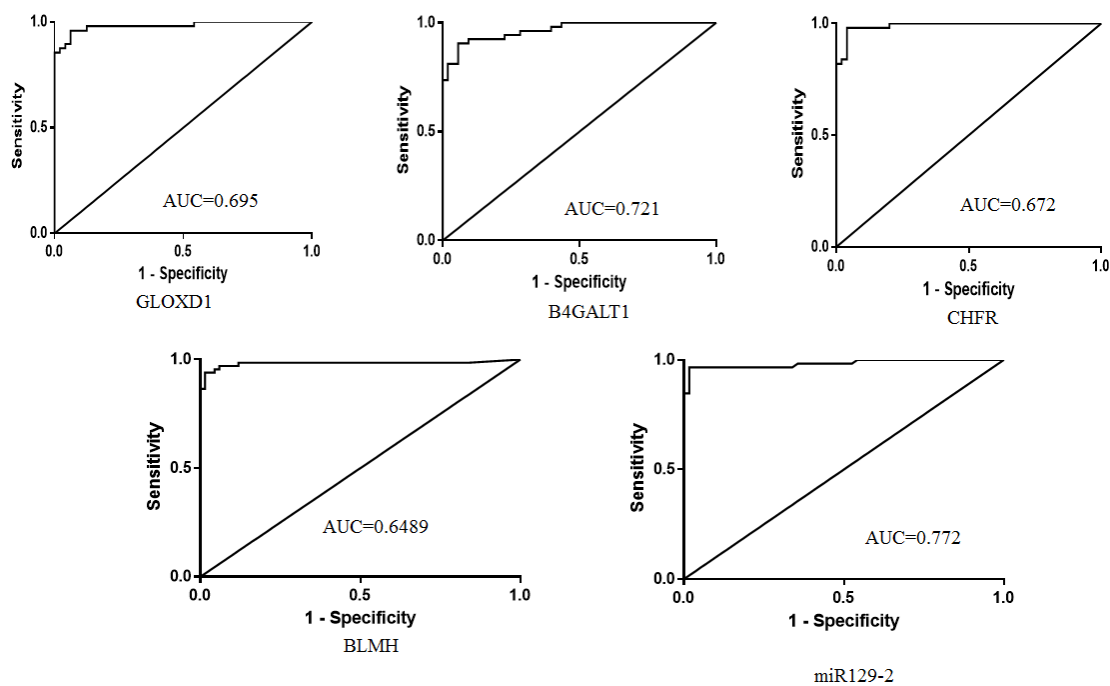


Figure. 5.8, Receiver-operating characteristics curves for individual gene analysis of DNA methylation levels in discriminating HCC from adjacent non-cancerous tissues.

5.3.5. Correlation with clinicopathologic parameters

For the evaluation of the biological significance of hypermethylation of these five genes in the HCC, we assessed the associations between the hyper-methylation status of these five genes and clinic-pathological parameters. In tumor samples, no correlation was found between the MPs of any genes and the clinicopathological parameters, such as patient age or gender, tumor differentiation and size, HBV and liver cirrhosis infection, tumor size and serum AFP levels of any five target genes.

5.4. Discussion

Hepatocellular Carcinoma is the most aggressive malignant tumor worldwide with a minimal outcome. Early detection and accurate discrimination between HCC and adjacent NTs may offer an opportunity to improve the long-term survival for HCC patients. Aberrant promoter methylation plays an important role in the process of tumorigenesis and biomarker discovery for disease diagnosis. To identify and characterize emerging markers in hepatocellular carcinoma, we used qMSP to evaluate a panel of genes among HCC and adjacent NTs. In this study, we decided to investigate the promoter methylation status of five genes (GLOXD1, B4GALT1, CHFR, BLMH, miR-129-2). These genes are involved in different molecular pathways of carcinogenesis such as cell proliferation (miR-129-2), apoptosis (B4GALT1 and CHFR). Understanding their functions in the advance tumor stages of HCC provides the ability to predict the premalignant conditions for early diagnosis.

The aberrant promoter methylation of numerous genes (GLOXD1, B4GALT1, CHFR, BLMH and miR-129-2) has been reported to be associated with liver carcinogenesis [24-32]. However, most of these studies evaluated the methylation status using non-quantitative methods. In this study, we performed quantitative methylation analysis for five selected TSGs. The qMSP method that we developed revealed that quantitative methylation analysis could provide more accurate and flexible information than non-quantitative analysis. For example, we could filter out low level background methylation by setting a proper cutoff value, and thus, improving clear discriminating between HCC and non-cancerous adjacent tissues. When compared sodium bisulfite based assays, qMSP is a rapid, easy-handling and cost-effective quantitative method that is particularly suitable for quantitative analysis of DNA methylation in clinical samples with a small amount of DNA. Several similar studies on qMSP, MSRE-based methods have also been reported. In a recent study, Moribe et al, D. Hua et al, Z-H. Huang et al [20, 23, and 33] reported that a qMSP, MSRE-based qPCR procedures were suitable in quantifying methylation levels on small amount of DNA (e.g., formalin-fixed and paraffin-embedded tissues). The major difference between our assay and the assays used by others are sodium bisulfite reaction by using the EZ DNA Methylation-Gold Kit (Zymo Research, Irvine, CA), cheaper qPCR fluorescent dye (Eva green) and a simpler calculation formula for MP in our method.

The five genes (GLOXD1, B4GALT1, CHFR, BLMH and miR-129-2) identified by qMSP as having the discriminatory power to identify HCC samples in this study affect different cellular signaling pathways. Among all five TSGs, the most frequently methylated genes were miR-129-2, GLOXD1 and CHFR, which are hypermethylated in more than 50% of HCCs. In addition, we found that 100% of the cases of HCC had at least one promoter

methyated in a panel of five targets (MiR-129-2, GLOXD1, CHFR, BLMH, and B4GALT1), suggesting that these genes may be potential biomarkers for HCC in the world populations. In Chinese populations, different combinations of methylation markers have been proposed for the diagnosis of HCC. APC, GSTP1, RASSF1A or SFRP1 showed 84.7% sensitivity and 87.8% specificity in discriminating between HCC and normal plasma samples and APC, GSTP1, RASSF1A, CDKN2A or RUNX3 showed 85.1% sensitivity and 89.4 specificity in discriminating between HCC and non-HCC tissues [23, 33]. The reasons for the discrepancies among studies may include different methodologies, samples types and patient populations.

Most HCCs are initiated as minute nodules. Dysplastic changes, which mark the transition to small, well differentiated HCCs, usually the nodules measure between 1 and 2 cm in maximum diameter. At this stage, tumors are easily detected by using various imaging techniques including CT-scan, MRI, and ultrasonography; however, it is not easy to discriminate pathologically between small size HCC and benign tumors such as dysplasia. The best way for an accurate diagnosis of this particular cancer may be to identify molecular changes that govern the transition to cancer. In this study, we found that the background liver tissues in HCC patients, where cirrhosis and chronic viral infection, also carried clear methylation of multiple genes when compared to adjacent non-tumor tissues, we also clearly detect tumor size I and II and discriminate with adjacent NT's tissues. The methylation analysis of these targets may not only be clinically useful in distinguishing HCC form adjacent NT's tissues but may also provide important information on the risk evaluation of HCC.

5.5. Conclusion

In conclusion, we examined the methylation levels of five genes in HCC and corresponding NT samples using modified qMSP method, and found that hypermethylation of a panel of three genes could efficiently distinguish HCC from adjacent NT samples. However, the value of these five genes or their combination for the diagnosis or risk evaluation of HCC needs to be further validated. In future, the quantitative methylation assays with an early stage of HCC samples will be required to gain new insights into our current findings and to identify an optimal combination of methylation markers with higher sensitivity and specificity for the diagnosis of HCC. The data from the present study showed that quantitative analysis of multiple methylated genes in tissue may be a promising tool for prognostic markers of HCC.

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CHAPTER VI

FUTURE RECOMMENDATIONS

6.1. Pharmacokinetic studies of anti-parasitic drug (BMCL26) in rat models

In this work, an LC-MS/MS method was developed for the determination of BMCL26 in rat plasma. The current work mainly focuses on the pharmacokinetic studies of BMCL26 in rat models. The pharmacological analysis of the drugs consists of two major components, pharmacokinetic studies and pharmacodynamic studies, which help to describe what the body does to a drug (PK), and what a drug does to/for the body (PD). Today, the pharmaceutical analysis is employed throughout the whole drug discovery and development process. It is used to provide accurate and precise data, not only supporting drug discovery and development but also post-market surveillance [1-4]. Pharmacokinetic-pharmacodynamic relationships are playing an increasingly important role in decisions on

the rational development and use of new drugs, and they can provide a detailed knowledge of the mechanism of the drug and a better understanding of the molecular targets on which they act. Due to the significance of pharmacological analysis, sensitive analytical methods are critically needed for pharmacological studies. Therefore, our long term goal is to provide guidelines in pharmacological studies of the anticancer drugs by quantitatively evaluating the molecular mechanisms of the drugs.

6.1.1. Experimental methods and design

The experimental protocol was approved by the ethical committee.

6.1.1.1. Animals

Male SPF Sprague-Dawley rats (240-280g, Charles River, Wilmington, MA) are used throughout the study. In addition, the pharmacokinetics profile varies between different animal species. Rats have been broadly used for safety and efficacy testing.

6.1.1.2. Number of rats:

A sample size of ten rats will be required in this research. In those ten rats four rats for training in anesthesia, drug administration, and blood sampling. One rat for testifies the dose of the drug. Then the optimized procedure will be used for a pharmacokinetic study on other five rats.

6.1.1.3. Drug administration

The compound is dosed via

1) Intravenous administration (IV)

2) Oral administration

The drug administration will be given by propylene glycol and saline (v/v 1:1). Depending on IC_{50} of the test compound, the compound dose will be calculated according to the following formula: $\{(IC_{50} / L * 10^{-3} * \text{molecular weight of compound/mole}) / (1 \text{ kg/L})$ when considering the body density of the Rat is roughly 1kg/L} multiple Blood samples (approximately 0.15ml) will be collected into heparinized-tubes before the dose and at 11 time points (at 0, 5, 15, 30 min, 1, 2, 4, 6, 8, 12, and 24 hr) after dose.

6.1.1.4. Multiple blood sampling

Blood samples (~ 0.2ml) are collected from tail vein cannula (if the cannulation failed, blood will be withdrawn from a lateral saphenous vein). Temporary cannulation of the lateral tail vein should be considered because multiple blood samplings are required over a short period of time as it avoids multiple needle entries without repeat damage to the tail vein. By using this temporary cannula, we reduce the number of needle entries and reduce the time the rat spends in a warming cabinet (since warming may not be necessary for taking blood via the temporary cannula). Tail bleeding normally requires the rats to be warmed in order to dilate the blood vessel prior to taking the sample.

No surgery is required. An intravenous catheter is inserted into the vein by puncturing the skin and taped in situ. A heparin flush is used (0.1 ml) after placement and between samples to prevent clotting. An access port is inserted into the exteriorized end of the cannula, which stops the blood from flowing, and the catheter is taped into place. Aseptic technique should be used. A local anesthetic cream (e.g. EMLA cream) can be applied to the site 30 minutes prior to insertion of the catheter. The tail may need to be washed with diluted Hibiscrub (1%) in order to see the blood vessel. The plasma samples will be obtained by centrifugation at 15000 rpm for 1 min and stored at -20°C until analysis.

6.1.2. Analysis:

6.1.2.1. Sample extraction

Protein precipitation (PPT) method will be used for the preparation of plasma samples because it is simple and fast. Plasma samples are removed from -20°C freezer and thawed to room temperature; the samples are deproteinized with 800 μl of HPLC grade Acetonitrile by vortex-mixing for 30 secs, and were ultra-sonicated for 15mins followed by centrifugation at $24,000 \times g$ for 15 min. The supernatant was transferred into auto sampler vials and the samples were analyzed using LC-MS/MS.

The PK analysis will be performed using Winnonlin program. For the multiple sampling methods, the analysis will be carried out on the plasma concentration time profiles obtained from each animal (n=3) (the concentration of CSUOHO901 in rat plasma on Y-axis and time on X-axis plot a graph for both routes of administrations). The PK parameter

calculations will be observed for maximum plasma concentration (C_{\max}), the time to reach maximum plasma concentration (T_{\max}), plasma half-life ($t_{1/2}$) and exposure of the compound calculated by the area under the curve (AUC_{last} and AUC_{inf}). For the comparison of the injection routes the major parameters of half-life [$t_{1/2}$], the volume of distribution (Vd), total plasma clearance (Cl) and area under the curve (AUC_{last} and AUC_{inf}) will be determined for each individual animal after multiple sampling to allow inter-animal comparisons.

6.1.2.2. Area under curve

AUC is an important parameter in pharmacokinetic studies; it can be presented graphically as the area under the plasma concentration (Y-axis) versus time curve (X-axis). AUC provides a measure how much and how long a drug stays in a body.

The Area under the Curve (AUC) is the area under the concentration/time curve calculated using the linear trapezoidal rule. In a single-dose study, the AUC (0- ∞) is usually calculated in two steps – the linear trapezoidal rule up to the last sampling point above the limit of quantitation, plus extrapolation to infinity.

6.1.2.3. T_{\max}

The time after administration of a drug when the maximum plasma concentration is reached.

6.1.2.4. C_{max}

C_{max} is the maximum observed concentration from time 0 to the last observed concentration for each subject.

6.1.2.5. T half-life

The half-life is the time taken for the plasma concentration to fall to half its original value.

Units for this parameter are units of time such as hour, minute, or day.

$$T_{1/2} = 0.693 / K_e$$

6.1.2.6. Volume of distribution [Vd]

The volume of distribution relates a concentration of drug measured in the blood to the total amount of the drug in the body. This mathematically determined value gives a rough indication of the overall distribution of the drug in the body.

$$V_d = \text{Amount of drug in the body at time } t (A_t) / C_{\text{plasma at time } t}$$

6.1.2.7. Clearance

Clearance can involve both metabolisms of the drug to a metabolite and excretion of the drug from the body.

In the broadest sense total (systemic) clearance is the clearance of the drug by all routes
total (systemic) clearance (Cl) can be calculated by either of the equations given below.

$$Cl = V_d \times K_e$$

Or

$$Cl = \text{Dose} / AUC$$

6.1.2.8. Bioavailability (F)

Bioavailability is defined as the fraction of the administered drug reaching the systemic circulation as intact drug. Bioavailability is highly dependent on both the route of administration and the drug formulation.

Subcutaneous, intramuscular, oral, rectal and other extravascular routes of administration require that the drug is absorbed first which can reduce bioavailability. The drug also may be subject to metabolism prior to reaching the systemic circulation, again potentially reducing bioavailability.

$$F = \text{Dose}_{i.v.} / \text{Dose}_{\text{other}} \times (AUC_{0-\infty})_{\text{other}} / (AUC_{0-\infty})_{i.v.}$$

6.2. Development of novel blood DNA test for early detection of hepatocellular carcinoma

Blood DNA test based on detecting tumor-associated DNA markers in blood is a highly promising method for HCC screening. Circulating tumor cells are detected in the blood

stream or lymphatic. Because blood circulates throughout the body and transports various cells, we have lesser biomarkers that are available for detection of HCC by using blood samples. The specificity and sensitivity of particular biomarkers are less, therefore by using blood samples we can identify the HCC in early stages (the tumor size is < 3mm) and investigate the methylation levels of multiple genes in hepatocellular carcinoma and to identify the best biomarkers combination from various biomarkers by sensitivity and specificity of genes. Recently biomarkers targeting tumor DNA in blood samples have shown clinical utility as a non-invasive “liquid biopsy” for solid tumors. For example, the plasma-based septin 9 (*SEPT9*) hypermethylation assay has been used clinically in colorectal cancer [11, 12]. Studies have also supported the use of a blood-based *INK4A*, *h19*, *IGF2*, *APC*, *P16*, and *PADI4* methylation assay as a biomarker for HCC [5-10]. The sensitivities and specificities are from 39.5 – 65.3 % and 65.3 – 87.2 %. In order to achieve the better sensitivity and specificity for circulating free DNA methylation analysis, we plan to combine the best single biomarkers and run as a single marker. For this work, we already detected better sensitivity and specificity panel of biomarkers on both HCC and normal tissue samples that can be used in future blood DNA test.

6.2.1. Experimental methods and design

6.2.1.1. Extraction of DNA from blood/plasma sample

Collect the blood/plasma samples form GLC biotechnologies laboratory. DNA is isolate from 600 µl plasma using TIA Namp Micro DNA kit following the manufactures protocol. To improve the extraction efficiency, carrier RNA will be added after the proteinase K

digestion. Plasma DNA is eluted in the final volume of 30 μ l sterile water and stored at -20 $^{\circ}$ C until use.

6.2.1.2. Sodium bisulfite conversion

Extracted DNA (200 ng to 1 μ g) has to be used for sodium-bisulfite treatment. Chemical modification of unmethylated cytosine to uracil within CpG Island will be performed using sodium-meta bisulfite treatment and its purification can be achieved by using EZ DNA Methylation-Gold kit. Then the bisulfite treated DNA will be collected by using M-Elution buffer (10mm of tris buffer) and stored in -20 $^{\circ}$ C. The modified DNA is used as a template for RT-PCR analysis.

6.2.1.3. Methylation analysis

Quantitative real-time PCR will be performed on Bio red CFX instrument. Bisulfite-converted standard DNA samples of know concentration is serial dilute and amplified using bisulfite converted ACTB primer. The standard curve prepared from this step is used for determining reference quantity of total DNA (Q_{total}) amount for each patient sample. Amplification will be performed in a 10 μ l reaction volume containing 4 μ l of modified DNA, 1 μ l of primer (biomarker), and 5 μ l of master mix (MgCl₂ solution, 10X PCR buffer, dNTPs and Taq polymerase). RT-PCR is conducting by using CFX96 Real-Time PCR detection system for 42 cycles, each of which consists of incubation at 95 $^{\circ}$ C for 10 min, denaturation at 95 $^{\circ}$ c for 20 secs, annealing at 66 $^{\circ}$ c for 30 sec, and extension at 72 $^{\circ}$ C for 45

sec. these three steps run 41 cycles than extension at 72 °C for 3 min, annealing at 95 °C for 1 min, extension at 60 °C for 1 min, followed by melting curve from 60 °C to 95°C read every 0.1 C hold. A positive control and a negative control (distilled water without DNA) are included for each of amplification. The specificity and sensitivity of each biomarker are analyzed by using Bio Red CFX manager software. Methylated percentage (MP) will be used to indicate the methylation level of target CpG sites at a specific location in the promoter. MP is calculating using the following equation:

$$MP = (Q_M / Q_{total}) \times 100\%$$

6.2.1.4. Statistical analysis

The difference between HCC and non-HCC patients will be analyzed using the Mann-Whitney U test or chi-square test where appropriate. Receiver-operating characteristic (ROC) curves will establish to determine a threshold value in distinguishing HCC and non-HCC and to determine the area under the curve (AUC).

6.2.1.4.1. Specificity and sensitivity of biomarkers

The specificity (likelihood of obtaining a negative result when the target is not present. or probability that a test result will be negative when the disease is not present) of the biomarkers assay by detecting the bisulfite-modified cancerous and noncancerous DNA through high-resolution-melting (HRM) analysis repeatedly; The melting temperature for each gene will be recorded as reference for the patient sample analysis. Sensitivity

(likelihood of obtaining a positive result when the target is actually present. or probability that a test result will be positive when the disease is present) of biomarkers will be determined by using the cancerous DNA

$$\text{Specificity} = (\text{True negative} / \text{Total number of samples without Cancer}) \times 100$$

$$\text{Sensitivity} = (\text{True positive} / \text{Total number of cancer with Cancer}) \times 100$$

6.2.1.4.2. Likelihood ratio

A final term sometimes used with reference to the utility of tests is the likelihood ratio. This is defined as how much more likely is it that a patient who tests positive has the disease compared with one who tests negative.

$$\text{Likelihood ratio} = \text{Sensitivity} / 1 - \text{Specificity}$$

6.2.1.4.3. Positive predictive value:

Positive predictive value of a test is a proportion that how likely it is that this patient does not have the disease given that the test result is positive.

$$\text{Positive predictive value} = (\text{True positive} / \text{true positive} + \text{false positive})$$

6.2.1.4.4. Negative predictive value:

Negative predictive value of a test is a proportion that how likely it is that this patient has patient has the disease given that the test result is positive.

Negative predictive value = (True negative / True negative + false negative)

6.2.1.4.5. Receiver operator characteristic curves

Receiver operator characteristic curves (so called because they were originally devised by radio receiver operators after the attack on Pearl Harbor to determine how the US radar had failed to detect the Japanese aircraft) are a plot of (1-specificity) of attesting on the X-axis against its sensitivity on the Y-axis for all possible cut-off points. The area under the curve (AUC) represents the overall accuracy of the test with a value approaching 1.0 indicating a high sensitive and specificity. If an AUC value approaching 0.5 indicating that sensitivity and specificity is not better than tossing a coin.

6.2.1.5. Combination of biomarkers

In this specific aim, we follow a similar method as the previous one which was used for the identification of better biomarkers combination in tissue samples. Using the above method, we identify the better biomarker combination for the early detection of the HCC in both cancerous and non-cancerous patients in serum samples.

6.3. References

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